ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

VOLUME 60, ART. 1 PAGES 1–182

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ROY WALDO MINER

SPECIATION AND VARIATION IN ASEXUAL FUNGI

BY

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NEW YORK
PUBLISHED BY THE ACADEMY
October 29, 1954

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(Founded in 1817)

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ROY WALDO MINER

SPECIATION AND VARIATION IN ASEXUAL FUNGI*

Conference Chairman and Consulting Editor

KENNETH B. RAPER

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* This series of papers is the result of a Conference on *Speciation and Variation of Asexual Fungi* held by the Section of Biology and the Division of Mycology of The New York Academy of Sciences, June 4, 1954.

INTRODUCTION

By Kenneth B. Raper
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In introducing this Monograph on *Speciation and Variation in the Asexual Fungi*, let me state that I do not believe that we are dealing with problems basically different from those faced by any investigator concerned with the taxonomy of microorganisms. In the so-called asexual fungi, however, these problems become particularly acute. The very fact that these organisms reproduce themselves so successfully without the intervention of sexuality at once precludes from consideration a developmental phase with attendant characteristics and structures which, if present, would normally provide the most stable features possible. In the absence of such features, we must somehow get along with what is left. That constitutes the problem which we all face, the problem on which we now focus our collective attention.

Let us recognize at the outset that we are not concerned with a natural group of microorganisms and, furthermore, that forms now considered as strictly asexual may graduate from this class at any time as we isolate additional strains or develop improved techniques for their cultivation and observation. To emphasize this point, I need only call your attention to the pioneering research of Dodge on *Neurospora* a quarter of a century ago and, more recently, to the brilliant work of Lederberg and others in demonstrating recombination in *Escherichia coli*. There are, of course, individuals who maintain that a stable taxonomy for any group of microorganisms is possible only when and if the perfect stages are known. To them I must say that knowledge of such stages would undoubtedly clarify our problems but, in the meantime, we must in some way handle the species problem in large groups of organisms which seemingly get along very well without this degree of completeness.

The question of what constitutes a species in *Streptomyces* is particularly troublesome at this time and, in the following papers, are presented a number of different approaches by individuals who are giving careful consideration to this group, including Burkholder, Duggar, Jones, and others. The task before them is admittedly difficult, but it is not unique and it is not unresolvable. Thom faced a comparable situation in *Aspergillus* and *Penicillium* a half century ago and, during the ensuing years, taxonomic schemes were evolved for these difficult genera which have met the twin tests of time and use. I shall not maintain that it is a simple matter accurately to identify a green *Penicillium*, but I do stoutly maintain that it is possible for any careful investigator to do so by following the recommended directions. We are most fortunate in having Doctor Thom present the story of how these schemes were evolved. Significantly, as more and more *Penicillia* and *Aspergilli* with ascosporic stages have been discovered, these perfect forms in the main, have been comfortably fitted into the schemes previously worked out for asexual stages only. Similar problems were faced in the equally difficult genus *Fusarium* by Hansen and Snyder, and they, too, have devised a rational scheme

for accommodating the plethora of molds which reproduce asexually in a *Fusarium*-like manner. As in the cases of *Aspergillus* and *Penicillium*, the discovery of perfect stages has tended to substantiate rather than negate the soundness of their taxonomic approach. We may be reluctant to accept the wholesale reduction in species which they advocate, but who else has examined a fraction of the material that has passed through their hands? Professor Snyder summarizes their work and tells us how they arrived at their present views regarding this difficult and phytopathologically important group of fungi. We shall see proposed a provocative scheme for classifying these and other economically important asexual fungi patterned somewhat after the systems that are now used for horticultural varieties of higher plants.

Turning to the bacteria, Doctor Buchanan will discuss the taxonomic problems faced by bacteriologists and recount the ways in which they strive to meet and elucidate them. His remarks should be particularly pertinent to our present problems, since current investigations, particularly those of Professor John N. Couch, seem to indicate a closer relationship of the Actinomycetes to the Bacteria than to any other known major group of microorganisms.

The necessity for examining microorganisms under carefully defined conditions of environment and nutriment becomes increasingly apparent, for only in this way can we accurately gauge the effect of external factors on the one hand and of inherent variability on the other. The presentations of Doctors Nickerson and McLeod, in particular, should serve to emphasize the importance of the former in affecting morphogenetic and cultural manifestations of microorganisms as these develop, while that of Doctor Stauffer should dramatize the tremendous variability that can be exhibited by a single mold strain when this is subjected to a succession and variety of mutagenic agents. In each case, the immediate effect may be to raise serious doubts regarding the adequacy of existing taxonomy. This is well and good. Is not the principal message, however, more constructive in character? Do not such studies alert us to the pitfalls inherent in attempting to make too narrow and restrictive the limits of the species which we recognize or describe?

This brings us to *Streptomyces* and the problems in taxonomy which this genus currently presents. Why should it be so troublesome? I shall not attempt an answer, but perhaps I may be permitted to record some observations. It is almost axiomatic that the ease with which a species of microorganisms can be recognized tends to vary inversely with the number of isolates available for observation and examination. If one has relatively few cultures, selected strains, or groups of strains, can, as a rule, be separated out as representing particular species. If one then obtains thousands of strains, as in the case of *Streptomyces*, intergradations between them tend to obliterate the lines of separation previously regarded as reliable. Instead of contributing, as they should, to a more stable taxonomy, the first impact is to confuse lines of demarcation which have previously been accepted. We now seem to be in this unsettled situation with regard to *Streptomyces*. Never before have so many representatives of one genus been isolated as in the case of *Streptomyces* during the last decade. The first impulse was to examine existing keys, note certain

differences in behavior, and then describe the isolate in question as a new species, particularly if it produced an antibiotic having hopeful possibilities. Today, this tendency seems to have abated somewhat, for, on the whole, investigators seem to be inquiring, "Is this actinomycete really different from all the previously described species"? If this impression is indeed true, it represents a most hopeful sign. In any case, the long view seems somewhat more optimistic. Time will winnow and sift the various characteristics (cultural, morphological, and physiological) which, today, are being weighed as suitable criteria for species separation, and the more significant and reliable of these criteria will undoubtedly be recognized and used. In the meantime, this process can be accelerated not so much by the numbers of strains examined but by the care and scholarship that is exercised in making and interpreting observations. We must know more about the extent and nature of variability inherent in particular species and strains, as Doctors Duggar and Backus are currently reporting. We must look carefully at differences and similarities in habits of growth, details of morphology, and capacities to utilize specific nutrilites. There are those who maintain that enzyme patterns are basic and, of course, genetically controlled. But do we have proof that the acquisition or loss of the capacity to use a certain carbon source represents a more deep-seated genetic change than is required to alter the pattern of colony growth or to effect some persistent structural modification in the spore-producing apparatus? Interpretations should be based only upon substantial bodies of reproducible data and then, I surmise, tempered with considerable caution.

Unfortunately, the situation in *Streptomyces* has been and continues to be complicated by considerations other than investigative and descriptive microbiology. Some of these organisms represent valuable pieces of property and, as such, they must be protected. To protect them and the products of their metabolism, patents must be obtained. So we are faced with the very difficult problem of being good sound microbiologists, on the one hand, while we attempt to provide materials for the patent attorneys on the other. I have been told that it is easier to secure a patent if the petition can claim production by a hitherto undiscovered species. If such is indeed the case, the clear need would seem to be not so much for more and more new species but for greater enlightenment of the legal mind. If we are to make real progress, we must address these problems as scientists, being guided only by the observations and interpretations which we can make with the tools and techniques of science and of sound scholarship.

The following papers represent an effort to accomplish just that end. We shall probably find different definitions as to what constitutes a species. We shall certainly see different approaches and methods employed in attempts to arrive at such definitions. More significantly, we shall exchange views relating to problems that are recurrent in our work. We shall probably settle little, if anything. But, if we can define a little more clearly the problems that are central to a better understanding of speciation and variation in microorganisms and, if we can bring these into sharper focus, our discussions will, I am sure, be eminently worth while.

SOME INTERRELATIONSHIPS OF SPECIATION, TYPE PRESERVATION, AND NOMENCLATURE IN BACTERIA

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Bacteriology, almost from its beginnings, has had an existence and development quite distinct from its mother science, botany. It was early recognized that bacteria constitute a group of asexual fungi, and that many of the criteria commonly employed in the systematic study of higher plants are quite inapplicable and misleading as applied to such fungi. These bacteria were found to have tremendous economic significance. This finding led to rapid development, recognition, and utilization of a whole series of new differential criteria. More recently, the pressure arising from knowledge of the economic significance of many of the cultivable (particularly the asexual) hyphomycetes has led to development of related criteria in a closely parallel path of systematic research on these forms. The organizers of the Conference on which this Monograph is based have therefore felt that there should be some review of the present situation in bacteriology.

A rational and satisfactory definition of *species* as a taxon in the class *Schizomycetes* is quite as difficult as the definition of species among the higher plants, and just as complex as in the asexual fungi. It seems rational and appropriate, therefore, that certain problems relating to speciation in the bacteria and in the asexual fungi should be considered together. Both bacteriologists and mycologists find that the answers given to problems of speciation by students of flowering plants, or of mammals or birds or insects, are in some cases helpful, but frequently quite inapplicable.

One issue should be faced squarely. Taxonomists interested in such groups as the vertebrates or the phanerogams appropriately stress sexuality and the taxonomic significance of intraspecific fertility and interspecific sterility. The progeny of the mating of the jack and the mare are infertile. Accordingly, the ass and the horse constitute distinct species. It would seem to be abundantly clear that these criteria are quite inapplicable to large groups of living things, such as the bacteria, the *Myxophyceae*, the asexual fungi, and the viruses. Nevertheless, there is reluctance to break with this concept even in these groups. The prevalence of the opinion is such that some comment on it seems desirable. One specific example may be cited of what would seem to be confused thinking.

A little more than a year ago there convened at this Academy a conference on the nomenclature and speciation of the viruses. Doctor Mayr, then ornithologist of the American Museum of Natural History, spoke on "Concepts of Classification and Nomenclature in Higher Organisms and Microorganisms." His address was an earnest effort to apply the concepts useful in species differentiation in vertebrate zoology to the viruses. One finds much difficulty in differentiating, in the material he presented, between concepts adaptable to microbiology and concepts having no practical application to it. Contrast

the present rapidly expanding taxonomic situation in bacteriology with the nomenclatural static situation in ornithology. Doctor Mayr stated "The last North American species of birds was discovered in 1889." He gives as his best definition "Species are groups of interbreeding natural populations that are reproductively isolated from other such groups." He admits that, in groups that multiply asexually, "The species concept is difficult to apply." He then makes the surprising assertion that "All such asexually reproducing lines will terminate sooner or later, either by extinction or by fusion with another line through a sexual process." He infers that sexual reproduction is merely latent in all those forms which we term asexual. He states that sexuality in viruses is nearly always found in "the cases in which it was seriously looked for." As a partial solution of the virological problem he suggests that we consider "all asexually reproducing descendants of a single sexual species as a 'collective species' to include all strains owing their origin to mutation (polyploids excepted)." Obviously, there would be imposed the obligation to trace this collective species back to a sexual progenitor. Finally, he agrees that "In practice it may be rather difficult to establish the relationships of such strains, and it may be necessary to adopt a provisional solution until the final relationship is established," and he further acknowledges that "the final decision will have to be based on an evaluation of the multiplicity of characters." Exactly so. With this last statement we can agree. After following a circuitous path and traversing much ground we have emerged from the maze exactly where we entered it. The experience may prove valuable in that two conclusions concerning speciation now seem justified:

(1) Concepts of speciation that may prove very useful in studies of higher forms may be wholly inapplicable to the groups of organisms with which we are at present concerned.

(2) If sexuality is discovered at any time in any of the organisms here regarded as asexual, this fact should be given due weight as another interesting and noteworthy differential character, but without material reevaluation of our recognized differential criteria.

In microbiology, we should profit by the experience of taxonomists of other groups, but we are essentially free to develop and characterize new differential criteria as we have the need.

Preliminary to any discussion of speciation or taxonomy in the bacteria, it would be wise to look carefully at some of the differential criteria which may be used in the diagnosis of species. In the case of higher plants and animals, the criteria (in addition to the reproductive gap) have been largely morphological resemblances and differences. These criteria are myriad, and seem, in general, to have been reasonably satisfactory in practice. But even in the characterization of these higher forms, we find, not infrequently, the inclusion of physiological (ecological) terms relating the plant or animal to its environment. Correlations between habitat and morphology that are more or less striking are frequently noted. Our taxonomic literature is full of such terms as aquatic, xerophytic, halophilic, parasitic, arboreal, heliotropic, and a host of others. In general, however, reliance has been based upon morphology. Physiology has been secondary.

The situation has been quite the reverse as regards bacteria. Just what are the categories of characters that have proved most useful in the diagnosis and differentiation of the various taxa of bacteria?

Morphology has proved useful but, in many cases, there are no readily detectable differences adequate to separate bacteria which, we are convinced on other grounds, are but distantly related. True, we have made good use of such characters as size, shape, sporulation, motility, flagella distribution, cell inclusions, capsules, grouping of cells, staining properties, pigmentation, colony morphology, sheaths, and many others. The usefulness of these criteria differs from group to group. The genus *Beggiatoa* may be rather completely diagnosed on the basis of morphology and the several species satisfactorily separated by microscopic appearance. But morphology alone proves quite inadequate for the separation of species, or even of genera, in enteric bacteria.

This inadequacy of morphology early led bacteriologists to employ physiology. We here define physiology to include all of the interrelationships and interreactions between an organism and its environment. While morphology can be studied in preserved specimens and microscopic preparations, physiology requires study of living organisms. Physiologists must study the effect of an organism on various environments and the influence of varying environments upon the organism. The effects on environment may be either physical or chemical, but the effect of the environment on the organism may be morphological or physiological, and changes so evoked may be either heritable or nonheritable. Changes in environment produced by a bacterium may also, in turn, stimulate changes in the organism itself.

The cells of bacteria are often far more complex physiologically than are the cells of higher plants and animals. There seems, in some cases, to be a high degree of negative correlation between complexity of cellular morphology and complexity of physiological activity. A morphological description of an organism may readily be made both adequate and complete. A physiological description of an organism may be made *adequate* by noting all characteristics useful in differentiation of the organism from related forms; but a *complete* physiological characterization of an organism would be so overwhelmingly complex as to be quite impossible, indeed, unthinkable.

Physical changes produced in the environment may include the production of light, the production of heat, and changes in viscosity and turbidity. Extracellular and intracellular enzymes of almost infinite variety are known to exist. The substrate attacked and the compounds formed are equally varied. The substrates may be broken down or built up to greater complexity. The relatively sudden access of new microtechniques for identification of compounds, and the use of paper chromatography have increased the ease of identification of products to the point where we are frankly embarrassed by our riches. We are possessed of an armamentarium of differential characters that would seem to be adequate.

The changes in the environment produced by an organism may in turn have a significant effect upon its morphology or physiology. Staling substances inhibitory to growth may be produced. Other substances may be formed which

trigger the mechanism that stimulates sporulation in a member of the genus *Bacillus*. Antibiotics of great variety, such as the colicines, may be formed. Special relationships such as symbiosis, antibiosis, and parasitism may be significant, and also the specificity of the pathogenesis and the lesions produced.

There have been innumerable attempts to arrange these changes induced by environment in the order of their importance and usefulness. None of these changes have any very wide spread differential applicability. Diagnosis of organisms in one group may be based upon ability to produce indole from tryptophane; in another, on the production of lactic acid from a particular sugar, or on the oxidation of free sulfur to sulfuric acid, or on the fixation of atmospheric nitrogen in a medium containing a suitable carbon source.

One should also note the effects of environments upon organisms and their significance in differentiation. Utilization of the multiple physical and chemical environments makes possible an almost infinite number of dichotomies and segregations. By the use of such criteria, bacteria have been placed in many categories. Specific and generic diagnoses may involve such varied elements as: need for or intolerance of free oxygen, the effect of radiant energy, salt tolerance, the effects of osmotic pressure of solutes and of physical pressure, utilization of the ammonium ion, the fixation of carbon dioxide, amino-acid requirements, special growth-factor requirements, the effects of antiseptics, of staling substances, and of antibiotics, the influence of enzymes such as lysozyme, the effect of hydrogen- and hydroxyl-ion concentrations, the effect of specific extractives in the phenomenon of transduction, and the significance of differential parasitism by bacteriophages. This partial list is sufficiently extensive to reveal in part the variety of physiological differential criteria available and in use.

The gamut of changes induced in bacteria is also extensive. There seems to be no need to list them here, but several general characterizations and observations may be in order.

The environmental element under study may be lethal or nonlethal. It may depress or accelerate growth. Optima, maxima, minima, and ranges may be observed. Changes observed in the organism may be morphological or physiological. The effect upon the organism may be transitory, in that change in environment may cause it to disappear or, much less frequently, a heritable change or mutation may be evoked. The importance of heritability of induced new characters in any study of speciation is obvious.

Mutations or saltations can be readily induced in bacteria and are commonly observed in the laboratory. But great care must be used in differentiating between environments which, on the one hand, merely sort out mutations already present ("spontaneously occurring") by creating a differentially favorable environment for the increase of mutant cells and, on the other hand, environments which seem rather directly to induce heritable change or mutation. To illustrate some of the complexities of the problem, we may cite one example long known.

One of the coliform bacteria (originally named *Bacterium coli mutabile*) ferments glucose actively with production of acid and gas. When lactose is

substituted for glucose there is a delayed reaction. After an "induction" period of some hours, the lactose is fermented with acid and gas. One may postulate several possible explanations of this "delayed" fermentation.

The induction period hypothetically might be the time required for the development of suitable *adaptive enzymes* catalyzing the fermentation of the lactose, or one might assume that, in the presence of lactose and under its influence, some cells may have mutated or adapted to the fermentation of lactose. The induction period in either case would represent, in part, the time needed for the increase of the lactose-fermenting progeny to a point where the fermentation is obvious. Or a third assumption may be made: that mutants are regularly produced by this organism quite independently of the presence of lactose, but that the few cells present remain submerged in the mass of non-lactose fermenters until, in the presence of lactose, they have a marked nutritive advantage. Singe cell isolation shows that the lactose fermenting strain is a mutant. Cultivation with glucose as the carbon source does not induce loss of lactose fermenting ability.

It is obvious that exposure to a new environment may (1) bring to light no change in a certain physiological reaction; (2) induce the development or significant increase in an adaptive enzyme which disappears in the absence of the stimulating enviornment; (3) induce the development of a mutant which may be isolated by suitable techniques and thereupon characterized; and (4) make conditions favorable for the relatively more rapid multiplication of submerged spontaneously developed mutants with consequent isolation and characterization.

Such variations or transformation in characteristics must be considered in any appraisal of speciation by the taxonomist.

One must also consider and appraise the influence of what may be termed "pigeon hole" differentiations and classifications, several of which have had and still have much use in consideration of problems of speciation and taxonomy. They should be characterized briefly.

One of these classifications, based upon fermentation of carbohydrates and related alcohols, has been widely (and rather helpfully) used in the differentiation and classification of the enteric bacteria. The postulates may be summarized and listed as follows:

(1) A strain of bacteria may fail to utilize or change a particular carbohydrate; or it may produce acid or gas, and the gas may consist of various mixtures of hydrogen and carbon dioxide. Any one of a half dozen or more other recognizable fermentation products may be formed.

(2) Each strain may be tested under suitable conditions with each of many carbohydrates and related alcohols.

(3) The strains may be characterized by listing the compounds attacked and the changes produced in each of them.

(4) If one utilizes numerous different available substrates and determines, for each, which of the several types of fermentation are shown, one may label a pigeon hole for each combination of characters. The number of pigeon holes available is determined by all the possible combinations of different elements. There may be many thousands or tens of thousands of such pigeon holes, all

distinct and duly labeled, certainly adequate for all purposes of differentiation. Schemata set up *in this form* have proved quite unworkable in some respects and for several reasons:

(a) Most of the pigeon holes remained empty. No organisms were found to occupy them.

(b) Many pigeon holes could be eliminated because of the correlation of fermentations.

(c) Many, indeed most, of those pigeon holes to which organisms were allocated showed such low correlation with the significance of the organism economically or scientifically that they were largely eliminated.

This carbohydrate pigeon hole system of characterization and arbitrary classification has been largely outgrown, but from it have been salvaged a number of fermentations which do correlate with other characters and are still listed among our important differential criteria. Note well that this fermentation schema, which is fundamentally a classification of certain cell constituents, is based upon the presence or absence of certain cellular enzymes.

Another elaborate schema (using the pigeon hole structure) is that propounded, frequently with enthusiasm, by serologists. It is based upon the presence of cell constituents which may behave as antigens and are therefore readily recognizable by serological techniques. One finds that these antigenic cell constituents may be grouped in various ways: some are somatic, some flagellar, some thermolabile, some relatively thermostable, and some occur only in smooth strains. Each may be identified, even in mixtures, by its reaction with specific antibodies. It is assumed that related strains (or species) may have, in part, identical antigens, and that there may be some significant degree of correlation between genetic relationship and the number of common antigens. Somewhat elaborate formulae composed of letters and of Arabic and Roman numerals have been devised to indicate the antigenic composition of the cells of each serotype. The antigens that have been recognized and labelled for strains of the genus *Salmonella* number several scores. One may readily construct from these data a series of pigeon holes, one for each possible combination of antigens. Such a schema would easily make provision for thousands of serotypes. Here again the schema tends (when fully expanded) to become unwieldy. In fact, the difficulties of its use in practice are so great that specially authorized laboratories have been set up for the identification of the serotypes already recognized and to assist in the characterization of new serotypes as these are isolated. All of this procedure seems appropriate and justifiable to the extent that the antigenic technique proves to be useful in the differentiation of organisms that are scientifically significant, or of practical importance in disease production, in sanitary science, in biochemistry, physiology, in the production or testing of antibiotics, and, particularly, in determining the epidemiology of disease outbreaks.

One point of terminology is that any strain having a definite known and identifiable antigenic formula recognizable by serological techniques should be known as a *serotype*. The problem of speciation in certain groups of bacteria is much complicated by the multiplicity of serotypes. Can they be combined into useful species?

The antigenic approach has brought to light many problems not yet adequately faced. It has been found possible to modify the antigenic complex of an organism by exposing it to certain cell extracts. One pneumococcic serotype may be modified by treatment with the extract of another. You will recall that the original small number of pneumococcus serotypes increased to unwieldy proportions. Maintainance of an elaborate antigenic classification was quite justified as long as successful antiserum treatment of pneumococcal pneumonia was determined by the serotype identified. The practical significance of serotype differentiation has been much decreased by the substitution of antibiotics for antisera. There is even some hint of a new pigeon hole classification based upon relative resistance to the several antibiotics.

A group of bacteriologists and enthusiastic virologists is now busily engaged in the development of a whole new classification of certain groups of bacteria based upon susceptibility of strains of the bacteria to lysis by various strains of bacteriophage. These phage strains are the virus counterparts of the physiological races of smuts and rusts. It is of interest to note that strains of bacteria are first used for the differentiation of the phage strains, and that the latter, when identified, are used in turn for the differentiation of bacteria. Much of value may well come out of these studies, though there seems to be no *a priori* reason for believing that phage susceptibility is a better criterion for species differentiation than the recognition of biotypes or serotypes. One may hope that reasonable effort will be used to correlate the phage findings with those secured by application of other criteria.

Bacteria, like the fungi, are readily induced to mutate. In fact, physiological mutants may be produced almost to specification and at will. The newer methods in which antibiotics are used to suppress the growth of "normal" strains have proved valuable in isolating strains in which one or more steps in a metabolic chain sequence of chemical changes have been eliminated and the nutrient requirements correspondingly modified. In some cases, it has been possible to combine two of these mutant strains. The complications of labeling and diagnosing and naming these forms will prove quite as formidable in practice as it has proved in the case of *Neurospora* or *Penicillium* or *Rubus* or *Quercus* or *Triticum*, although in the cultivable fungi and bacteria we have the advantage that it is always possible more readily to control environment of the living culture.

All these considerations lead to the conclusion that, in the case of bacteria (as also in asexual fungi), the problems of speciation are in many respects more difficult and, in other respects, less difficult, than in the higher plants and animals. I believe that the following conclusions and comments are justified:

(1) The principal criteria used for the differentiation of species (as well as of other taxa) in higher plants and animals are based on morphology. This inference means that reference material (type specimens) can be dried, or preserved in other ways, in herbaria, exsicatti, collections, and museums.

(2) Smaller living things, particularly the filamentous fungi and especially the bacteria and viruses, usually cannot be differentiated satisfactorily on the basis of morphology alone. Physiology frequently furnishes a more important basis. This finding means that all reference material must be kept alive and

in such condition that it can be grown at will and its environmental reactions observed. The well-kept culture collection must, in general, replace the herbarium and the museum for the forms studied. Mayr, in a list of three desiderata in nomenclature, well says "Every name, whether of a lower or of a higher category, must be clearly based on an objective type. Methods of investigation are continuously changing and, unless objective types are deposited, it is impossible to re-examine at a later date the unit on which a name is based." Such re-examination of an objective type requires living material.

(3) The codes of nomenclature of botany, bacteriology, and zoology emphasize the importance of the type concept in the determination of the species. Those who phrased the wording of the codes had generally in mind the preservation (usually in dried form) of the actual specimen on which the original descriptions were based. As noted above, the type specimen of a virus or a bacterium, whenever physiological characters are included as differential criteria, must be in the form of a living culture derived from the one from which the original description was derived. If the original type culture has been lost, a culture agreed upon as a standard or substitute type may be substituted.

(4) A *species* of any organism which must be diagnosed, in whole or in part, by inclusion of physiological characters is *the living culture of the organism from which the original description was drawn, together with other cultures considered to be sufficiently closely related.* The type culture is a central point to which bacteriologists relate all other cultures believed to be allied. It is a further requirement that the type culture should be maintained and grown under such environmental conditions that no significant change of characters occurs.

(5) Type cultures of all available species of microorganisms should be maintained by national or international collections of type cultures. In the United States, we have the American Type Culture Collection at Washington, D. C., under the supervision of a board of trustees chosen by the several microbiological societies and by the National Research Council. There are several other culture collections of viruses, fungi, and algae in the United States which exchange material freely, and corresponding collections in other countries with which excellent relations are maintained.

(6) Not only should our type culture collection maintain, as far as practicable, the types of each well-authenticated species of microorganism (or, in the absence of a true type culture, a substitute or standard culture which has been internationally approved) but also, again as far as practicable, strains of important organisms of subspecific rank, which should constitute the types of these subspecific taxa, as subspecies, varieties, clones, mutants, serotypes, *etc.* Many of these taxa are of material, economic, or scientific interest. They are useful in disease diagnosis, as tools in biochemistry, in studies of physiology, in cytology, in the production of antibiotics, in the manufacture of organic chemicals, in the evaluation of antiseptics and disinfectants, in the production of antisera used in typing, in studies of heredity, *etc.*

(7) A type culture collection should be an active research center, carrying on studies at the Center and working with specialists actively in the monographing of the several groups. Unfortunately most students, when describing

new organisms or new strains on which they are working, emphasize those characteristics in which they are directly interested, and fail to differentiate the organisms satisfactorily from other related forms. A paper on new organic chemical compounds, before acceptance by a chemical journal, must characterize the compound so that it can be recognized by others. Many of our bacteriological efforts at characterization are amateurish in comparison with those of the chemist.

One eventual research task of an efficient type culture collection is the adequate description of the cultures maintained. When a subculture is secured by an investigator, its history and characteristics should be made known and furnished with the culture.

Another research task is the study of methods which will insure the maintenance of living cultures with a minimum of variation or change. Techniques for accomplishing this end have greatly advanced in recent years, but much work in this field remains to be done.

(8) Recognition and differentiation and identification of bacterial species and other bacterial taxa must continue to rest upon several bases:

(a) Classification and organization of taxa must change with increasing knowledge and use of new and advanced techniques. There should be no effort, at present, to crystallize findings into permanent form.

(b) Classification and taxonomy should continue to attempt to be natural, endeavoring to indicate real genetic relationships. While this object is our goal, we must realize that, in many groups, we are far, as yet, from having or knowing the really significant differential criteria.

(c) All classifications and taxonomy and, particularly, nomenclature in microbiology, must be highly utilitarian. The bacteria and related forms, their names and classifications, are utilized for many purposes by many individuals, including medical men, veterinarians, biochemists, zymologists, analytical chemists, nutritionists, students of animal and plant physiology, plant pathologists, geneticists, soil technologists, sanitarians. These workers, and many more, demand stability and common sense in the classification and naming of microorganisms.

(9) Gradually, the names used for the various cultures in our type culture collection should be studied and fixed by international agreement where there are differences in usage at present. This proposal does not mean that change should not come with advancing knowledge.

The bacteriologists, through agreements at International Congresses, have set up an International Committee on Bacteriological Nomenclature which has a membership at present of about 100, including representation from the microbiological societies of about 30 nations. This body has, as an executive committee, a Judicial Commission authorized to pass upon many problems of bacteriological nomenclature subject to review by the International Committee upon appeal.

The botanists have also set up a liaison committee having to do with bacteria, and a permanent committee dealing with problems of nomenclature in the fungi. There is abundant reason why bacteriologists and mycologists should work together on the nomenclature of groups with which both are concerned.

(10) Some special emphasis should be laid on the economic significance of decisions relating to speciation and on the nomenclature of the taxa. We have techniques of differentiation of such great variety that it would be possible to recognize literally millions of species and subdivisions of species of bacteria. Such complexity will not be tolerated by practical workers.

VARIATION AND SPECIATION IN THE GENUS *FUSARIUM**

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For 150 years, since the genus *Fusarium* was first described, man has puzzled over the problem of identification and classification of *Fusarium* species. By no means has *Fusarium* been unique in this respect, but the world-wide distribution of the genus and its great economic importance have demanded exceptional attention. Its ubiquitous occurrence in soil, water, and on all sorts of substrates and, especially, its role in the destruction of wild and cultivated plants have led to claims that *Fusarium* causes more damage to world agriculture than any other genus of the fungi. Whether or not these claims are true, they serve to make clear why plant pathologists and microbiologists, out of the sheer necessity of having to deal with such economic fungi, have required a practicable and dependable system by which *Fusarium* species may be classified and labeled.

Most persons would agree that progress has been made in this direction. Whatever one's concept of species may be, or whatever system of *Fusarium* taxonomy one may follow, certainly the identification of *Fusarium* species is more rapid and more certain today than at any previous time. Unfortunately, this cannot be said for most other genera of fungi. Yet the knowledge of fungi gained directly and indirectly in the first half of the 20th century, particularly through research in the fields of plant pathology and genetics, has provided a basis upon which one can designate species with far greater confidence and on a much sounder scientific basis than has been previously possible.

It is our intention here to present the kind of experimental evidence and logic upon which we have arrived at the conclusion that only nine species can be justified in the genus *Fusarium*, and to offer a concept of species and a system of nomenclature which, we are convinced, holds considerable promise in meeting the problem of speciation not only in *Fusarium* but also in other fungi, whether they be sexually perfect or imperfect organisms.

Historical Background

The first hundred years of *Fusarium* history or, roughly, the 19th century, was a period in which *Fusaria* were collected and named but, for the most part, not studied in the modern sense. This period was one of accumulation of the individuals of a genus. Hundreds upon hundreds of collections were made, each usually represented only a few stems or leaves or seeds, upon which were the spores of a *Fusarium* or *Fusarium*-like fungus. On the basis of very superficial observations on the spores and upon features of appearance and habitat, species were named and described. Almost never were cultures made and, more frequently than not, the descriptions were scant and inadequate. This era was one in which the systematist looked not so much for similarities between the individuals of a species as for differences of any kind, no matter how

* Presentation of this paper was made possible by the generosity of The New York Academy of Sciences, during the senior author's tenure of a European travel grant from the Rockefeller Foundation.

minute, upon which to base the description of a new species. Most species of this era were based inevitably upon a few parts of an organism, not upon the whole organism, because these fungus collections were not cultivated. Because they were not cultured, neither the polymorphism nor the variability of fungi were recognized. It becomes evident, then, that the naturalists and systematists of that era were naming species mostly on nonliving fragments of an organism, and that such species were necessarily of a relatively fixed and narrow kind.

The great stimulus given to *Fusarium* taxonomy, near the close of this 100-year period, was provided by plant pathologists for economic reasons. When pathologists began to demonstrate, by means of experimental studies, that *Fusarium* was in fact the actual cause of much of the crop loss with which early collectors of the genus had found it associated, they themselves took up the problems of speciation and naming. Appel and Wollenweber's *Grundlagen*, published in 1910 in Germany, laid the foundation for all later studies on the subject. Wollenweber continued to work with the genus to the end of his life, and the monumental series of monographic treatments published by him during his lifetime constitute a remarkable contribution to the field. Many others, including Sherbakoff, Brown and his group, Reinking, Padwick and his students, Gordon, and we ourselves have introduced new experimental evidence resulting in different viewpoints. In spite of the progress which has been made, however, adjustments and changes will continue to take place in the future, as must always be the case in the taxonomic field when new facts and new interpretations make revision necessary.

Recognition of Variability

The most significant advance which has taken place in the first half of this 20th century as regards our knowledge of the fungi is the experimentally proved concept of variability. This advance has been made principally by the plant pathologist who, confronted with a variety of cultural or behavior expressions in the etiology of disease, has had to determine whether a given disease has been incited by a complex of fungus species or by several variants of one species.

Variability has been established beyond question in one organism after another, in all classes of the fungi. Experimental data however, continue to accumulate with each organism studied, and currently such information is being gathered extensively as regards the genus *Streptomyces*. One may now say that variability in organisms is a principle not only of microbiology but of all biology. Yet one must not forget that, during the last century, when the foundations for present day mycology were being laid, mycologists did not have the benefit of this principle. And although the principle is recognized and accepted today, the significance of variability is not yet fully appreciated, nor is it widely utilized. Systematists especially have been slow to apply the knowledge of variability in the taxonomy of fungi. In the *Fusaria*, Wollenweber first reduced the number of species to about 600, but gradually, as the variability concept gained headway, he reduced this number, in 1935, to about 65 species and 55 varieties. However, even though he accepted the principle that fungi are variable, he did not use the single spore culture method, nor did

he analyze species for the breadth of variability. Therefore, like most systematists in this and earlier periods, he did not know *how* variable fungi can be. We must realize, then, that great as the contributions of these earlier systematists have been, they worked without the benefit of this most important 20th-century concept of variability.

Our approach to the taxonomy of *Fusarium* has been the determination of the *extent* of variability. This approach has involved the collection of individual specimens from nature and from laboratories throughout the world, and the analysis of genetic variability in each. Many variants were secured directly from nature, others were obtained in the laboratory. When these were studied morphologically, it soon became evident which characters were not suitable and which were stable enough to serve as taxonomic criteria.

The first group we worked with was that known in Wollenweber's classification as section Elegans, containing 10 species and several varieties and forms. No perfect state has been found for any member of this section. This group of *Fusaria* led us into taxonomic studies because it was one of the most important groups from the standpoint of plant pathology and because these *Fusaria* were among the most difficult to identify. We became involved in the problem of identifying them simply because it had become necessary to deal with these fungi as pathogens of agricultural crops. In analyses made of the variability in a large collection of these fungi, using single spore techniques, we found that one individual frequently would exhibit the capacity to vary so broadly as to encompass the other species of the section. The analyses showed also that spore size, septation, and sclerotium formation were not dependable characters for speciation. Not even the ability to produce sporodochia, a family character, was consistently shown in the progeny of a single spore. These findings left us with no alternative other than to recognize all members of section Elegans as one, large, variable species, *Fusarium oxysporum*, having rather well-defined characters of spore shape, and composed of many biologically distinct formae as measured by their pathogenesis for various host plants.

How do we know we were on sound ground in following this procedure? There was no sexual stage by means of which to prove inheritance of the characters involved. The answer to this question was obtained in working with another group of the *Fusaria*, now known as *Fusarium solani*.

The extent of variability in the imperfect species, *Fusarium solani*, has been illustrated in a striking manner by the analysis of the genetic capacity for variation exhibited by the progeny of one perfect individual of the species. From one perithecium were prepared a number of single ascospore cultures, and the variants of these were collected. These variants, in turn, were mated with each other and with the parent type, and their progenies collected. Finally, from this one individual, we have arrived at an approximate picture of natural variability in respect to colony color, configuration, rate of growth, topography, relative abundance of conidia, presence or absence of sporodochia and of sclerotia, size of spores, their septation frequency, and even their pathogenicity. This analysis showed which characters could be used to delimit the species and which could not be so used. But first of all, it became clearly evi-

dent that each variant, because it was genetically and culturally different, must either be named a separate species, or that all these genetically different individuals must be accepted for what they are, the individuals and clones that comprise one large and variable species.

This decision led us to the second step in the delimitation of a species, that of the synthesis of the species.

Synthesis of Species

The delimitation of a modern species may be divided into three steps. The first step is the *accumulation* of the individuals which come into question. These may be the available members of a genus or all of the individuals which may appear to be related and their progenies. The second step is that of *analysis*. An attempt is here made, by means of experimental procedures, to determine the limits of genetic and apparent variability, especially in respect to morphologic features, since these limits inevitably must provide the critical criteria for all future species. The final step is that of the *synthesis* of the species. This step involves the judgment of the investigator, for it is here that one must judge, from the analytical data available, which morphological characters are common to all individuals of the species, and which serve also to distinguish all the individuals of this species from all the individuals of other species. It is a matter of judgment whether or not the characters selected for the delimitation of species are practicable, and whether or not the average biologist who wishes to determine species in the genus concerned will find it feasible to do so with the facilities and training at his command.

In *Fusarium solani*, a species in which all of the variants and progenies from a single individual were studied, it was found that there were many striking differences between individuals in such features as colony appearance, color, rate of growth, spore size, *etc.*, but these characteristics only served to emphasize that it is not the differences between individuals which provide the bases for speciation but rather the similarities which tie them all together. In this species, the shape but not the size of the macroconidia was consistently so similar that this character provided a most useful and ever present character upon which to base the species, since macroconidia presumably must always be present, in order to recognize a fungus as a *Fusarium*. Companion characters in the determination of this species are the microconidia and the chlamydospores, their presence and their kind.

The test of satisfactorily identifying a species is the convenience and certainty with which biologists may recognize its component individuals. It would appear now that *F. solani* and *F. oxysporum*, the two species in the genus most commonly isolated by plant pathologists throughout the world and the two species which contain the greatest number of biologically and economically important forms have met this test. Yet as late as the 1930's, these two species, then known under the names of dozens of different species and varieties into which they were divided, were found to be a cause of constant confusion by the plant pathologist.

In the taxonomic treatment of the remainder of the genus *Fusarium*, we have followed the same procedure of accumulating as many individuals as

practicable, analyzing them for variability and then synthesizing species on the basis of the most dependable and usable morphologic characters. Instead of 1500 to 2000 names, we accordingly recognize only nine species and no varieties in the entire genus. We designate with the third term the *forma specialis*, of a trinomial, the approximately 40 biologically or pathogenically distinct intraspecific groups of individuals where it is important or convenient to man to do so.

Speciation

If biologists or, particularly, mycologists, can not agree upon a definition for species, perhaps they can agree more easily upon what a species is not. If so, then mycologists thereby might come to closer agreement, indirectly, on a concept of species.

It would seem to us that one of the precepts of taxonomy is that organisms be identifiable to species on the basis of what they are, not upon where they occur or what they do. Furthermore, that taxonomy should aim to make it possible to determine the species, whether the organism be dead or alive, providing the morphological features used in its classification be present. These requirements necessitate that the species be morphological. The species category has been established as a morphologic one, and the more knowledge we gain, the sounder the concept of the morphologic species becomes. If mycologists could agree to abide by this experience of most biologists, we should then reject in practice, as well as in principle, the physiologic or biologic species and find ourselves thereby in good agreement on the basic issue, that the species be a morphologic category. Pathogenicity, the production of a certain biochemical or group of biochemicals, or any other feature of physiologic behavior might be designated then by terms indicating intraspecific races, formae speciales, or clones.

Another precept in taxonomy should be, in our opinion, that valid species be readily identifiable. If a person trained in biology can not readily determine the species to which an individual belongs, in most cases, then there is something wrong with the taxonomic system, not with the fungus! In other words, the species in particular but taxonomy in general must be designed for convenience and practicability. If it is not usable on this basis then it does not justify its existence.

Also it should be a precept in taxonomy that a biologist must know his species before he describes it. To know it means, in most cases, to cultivate it in pure culture, if culturable, or to grow it upon its host if not culturable, and to know its morphologic features on suitable substrates under a variety of conditions, and especially to know something of its capacity for morphologic variability.

When only a few individuals are the subject of a taxonomic study, it is easy to distinguish between them, and these individuals are therefore often named as different species. But when many isolates are assembled it becomes much more difficult to distinguish between the first-named species. This difficulty usually means that they are all individuals of the same species, and that the differences first seized upon were not valid as species criteria. The more

isolates there are of a species, the easier it should be to recognize a species. Such species are usually large, and may well appear to be natural groups. We consider our *F. solani* as a rather natural, well delimited species, relatively easy to recognize, and this description applies to the mutants or variants obtained in the laboratory or in nature (and, contrary to general belief, variants are detectable in nature just as they are in the laboratory).

"*Horticultural Varieties*" *in Fungi*

Much of the disagreement among mycologists on the subject of species centers around the question "are the features exhibited by this group of individuals a sufficient basis for speciation?" All may agree that the group of individuals in question shows some distinctive characteristics, but all may not agree that these characteristics are distinctive enough to be described in such a way that other mycologists will recognize readily the individuals of that species from a description. Do these individuals represent merely an intraspecific group which is, perhaps, of some economic importance and is therefore proposed by some investigators as a species?

This kind of problem is not unique with the fungi. In *Pyrus malus* and *Pyrus communis* we have come to recognize rather distinct intraspecific groups now known as horticultural varieties. No one denies that such varieties show morphological and other characteristics recognizable by experts, nor is there any longer any serious thought of making species of such horticultural varieties. *Solanum tuberosum* is another well-known instance where a clone, or a group of clones, becomes established as a horticultural variety because of certain distinctive features of economic importance, which make it desirable to name it while still recognizing that the characteristics are not suitable for speciation. So it is with many species of higher plants with which we have become most familiar because they have become in some way important to man. In particular cases, where great economic value, or potential value is attributed to the horticultural variety, as in new varieties of roses, the horticultural variety enjoys legal status and may be patented.

Do we not have an analogous situation in the fungi? As we become more familiar with the constituents of a fungus species, economically important because they destroy crops or create a valuable biochemical, we come to recognize them sooner or later as clones, the equivalent of the horticultural variety. Either these divisions are selected in nature or, in the case of the production of biochemicals, fungi are bred, or selected in one way or another to develop a superior quality and yield of a given product. What else is this process than the employment of the same principles and techniques we have been using for years to develop horticultural varieties of higher plants?

As far as we know, the horticultural variety or its equivalent (whatever one may call it in the case of the fungi), has never been used for the fungi. Its use was first applied, to our knowledge, in an unpublished manuscript written jointly with J. W. Oswald to meet the problem of handling distinctive clones of *Fusarium roseum*, formerly known as different species. It is now planned to publish this manuscript, as a proposal for the use of this kind of variety in the fungi. It will suffice here to illustrate its use in another *Fusarium* species,

Fusarium oxysporum. We have combined into this species the 10 species and varieties of Wollenweber's section Elegans. One of the 10 species, namely *F. redolens*, is currently considered by some to be sufficiently distinct to warrant its recognition as a separate species. We agree that there are some features of *F. redolens* that are recognizable by a specialist of the genus *Fusarium*, but also we are convinced that these differences are comparable only to those of a horticultural variety within the species *F. oxysporum*. We accordingly propose to designate this former species as follows: *Fusarium oxysporum* "Redolens," just as one might designate the horticultural variety of peas, known as Perfection, as *Pisum sativum* "Perfection."

The utilization of the horticultural variety concept in fungi provides a device for the recognition of subspecific clones, or groups, of a species in a manner in which we have been long accustomed, in higher plants.

Discussion

Researches made upon fungi since the beginning of the 20th century, in the fields of plant pathology and genetics, have established beyond all doubt that fungi are variable. But how variable? Apparently more variable than most systematists realize! This variability extends more or less to all characters so far studied, whether, in respect to morphology or to physiology, defined here to include pathogenesis and the production of biochemicals. This concept of wide variability has brought about a revolution in mycological thinking. When applied to the field of taxonomy, it is seen to require revisions of most of our fungus classifications toward a smaller number of genera and species.

The fact of wide variability emphasizes more than ever the necessity of morphologic species. Morphology is more usable and more dependable, and therefore more suited to speciation than are physiologic characters. Yet morphologic differences between individuals, no matter how striking, are not in themselves a justification for naming species. Only an analysis of variability will show which characters are common to the individuals of a species and which are not.

The significance of variability in terms of classification is that future species will be larger and will be morphologic, and that biologists who would describe a new species will find it essential to determine first its range of variability.

With the trend in taxonomy to larger species, and the designation of greater numbers of intraspecific categories, it becomes increasingly evident that taxonomic revisions will necessitate changes in nomenclature and in the *Rules of Nomenclature*. We have too many subspecific categories, and they are inadequately defined. We question the need for any categories below the morphologic species level other than forma specialis, race, and clone. These divisions, with the use of the "horticultural variety," would seem to take care of the needs of fungus classification in most cases. There are other problems in nomenclature which will be receiving increased attention by mycologists interested in economic fungi, such as modification of the type species concept, the questionable practise of using different species epithets in the perfect and imperfect states for one and the same fungus, and name changing without taxonomic advance as now sanctioned by the rule of priority.

Changes in taxonomy and nomenclature necessitated by advances in knowledge of the fungi take place slowly. They will be speeded up only to the extent that those who work with economic fungi want changes made and see to it themselves that appropriate steps are taken in this direction.

Summary

Although there are between 1000 and 2000 names proposed in the literature for species and varieties of the imperfect genus *Fusarium*, we contend, on the basis of experimental studies, that only nine species are justified on a morphological basis. This conclusion has been arrived at by analyses of variability under various environmental and cultural conditions, using the single spore technique, and by the synthesis of morphological species based only upon those characters which are the most stable. These analyses, by showing what a species is not, have provided a better basis upon which to judge what a species is. Physiological characters are regarded here as suited only for the delimitation of intraspecific categories such as the forma specialis, race, and clone. Such a concept of speciation is believed to be in full agreement with 20th-century knowledge of fungus genetics and variability, but is obviously at odds with 19th-century concepts of narrow and relatively fixed species upon which present-day mycology is founded. The 20th-century facts on the behavior of fungi have developed at an amazing rate, while taxonomy and nomenclature have lagged far behind. Yet taxonomy and nomenclature comprise the essential framework into which our knowledge of fungi must be arranged and catalogued. We propose to meet this challenge by a classification of fungi based upon large, morphological species, and by the use of a simplified nomenclature better suited to the cataloguing of the various intraspecific divisions. It is believed that these modifications may offer a sounder taxonomy in mycology and, at the same time, provide a method of naming fungi which would be adapted to the labeling of economic fungi.

THE EVOLUTION OF SPECIES CONCEPTS IN *ASPERGILLUS AND PENICILLIUM*

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Why should we have speciation in asexual fungi?

Disregarding theoretic taxonomic considerations, molds have become so important in human affairs within the past 50 years that some dependable mode of identification has become necessary. Investigators who have faced this problem during this period have been fully aware of the unimportance attached to their study by individuals concerned with theoretic systematic cryptogamic botany. Some of us can remember when molds were unceremoniously dumped into what were opprobriously designated "form" genera, based upon crude examination, with names marked to be discarded if and when anyone looked at them long enough to find where they belonged.

Certain basic statements more or less generally accepted in this connection may be set down:

(1) Sex: some "perfect" form either is discoverable or may be assumed to exist for each of the types of structure formed.

(2) From the geneticists' viewpoint, if and when they look at all, the molds we study are assumed to be haplonts doomed to be celibate only until the other mating type is found.

(3) The rigid interpreter of rules would change the generic name in use to any name concocted for one perfect strain.

(4) The industrial user will continue to ignore this technicality as impractical.

(5) The organism found useful is entitled to a name that is definite, recognizable, and not confusing.

The task assigned to me at the Conference on which this Monograph is based called for a report on the background and course of development of the current taxonomic situation in *Penicillium* and *Aspergillus* under both industrial and academic research conditions surrounding that evolution. Before I met this problem I had been in some great phanerogamic herbaria and was well acquainted with type specimens and priority claims.

My first contact with the task of identifying a fungus important in an industrial process occurred 50 years ago (March 1904) and first reached definite publication in 1906. At the very beginning, I faced the demand from industry that there must be a Latin name for the ripening agent of the cheeses involved. The demand still stands throughout industry (and, dare I add, throughout chemistry also). Even now a finder of a *Streptomyces*, which seems to him interesting, gives it a Latin name. There is, on the one hand, unconcealed contempt for taxonomy; while, on the other hand, a Latin name followed by a few lines of vaguely assorted technical terms adds academic flavor to the mystery and seems essential in the Patent Office.

For my purpose, the cheese literature was searched and found hopeless. The mycological literature did not help me. Some of the great herbaria were

visited. The fragile and ephemeral bodies of *Penicillia*, some *Aspergilli* and many other molds furnished few type specimens of value. These asexual forms were shunned in the academic laboratory as members of "form" genera and were scarcely interesting academically. Meanwhile, the increasing recognition that the very same molds were important in industry created a demand for some way to compare our results and understand each other's reports.

Under such pressure, we turned to laboratory culture. Many others did the same thing. Each followed his own ideas, however, and described his material accordingly. Search for the optimum culture medium occupied many minds, only to leave us without comparative data. Accordingly, step by step, we were driven to compare great numbers of isolates which appeared to belong to some particular genus, and to do so under definitely unified conditions. As regards the group of fungi with which we were concerned, we were compelled to concentrate our attention on one nutrient substratum, and that substratum had to be one which could be standardized and duplicated by any worker anywhere. Exploratory study of every strain under other conditions gives added and often important data, but the absolute essential is the measurement of what each isolate of a series will do when put in actual comparison with its fellows under sharply defined uniform conditions.

We are keenly aware that many of the strains we meet are imperfect fungi. We have been lectured repeatedly that we must search to the utmost for the perfect form. In one case, we got a clue. We knew what to look for, and we overhauled every strain of that series that came into the laboratory for five years without success. Then a girl working in an industrial laboratory in England found such a strain in a bottle of pickles. Yet we have seen that asexual thing from places in Manchuria, China, Africa, various areas in Europe, and in the Americas. We have not heard of another perfect strain. We had that ascosporic strain for years, and it never escaped to plague anyone as a contaminant. There are whole series of successful molds, world-wide in their distribution, nearly omnivorous in their nutritive demands, found essentially wherever man goes, which have so far cut loose from any so-called perfect form, without which they get on comfortably. Then we find a thing like *Aspergillus fumigatus*, also found everywhere and on anything, while much less commonly we find the ascosporic *A. nscheri*, with fumigatuslike conidial heads, persisting but less frequently, so that their existence went unreported by the great laboratories for 50 years after *A. fumigatus* was well known. No one has ever induced the common *A. fumigatus* to produce ascospores. I am not saying that haplonts completing the story for *Penicillium roqueforti*, or *P. exansum*, *Aspergillus niger*, or *A. flavus* will not be found. What I am saying is that these species, as we see them, have so prospered without a trace of sex that, for practical purposes, they have discarded that phase of their life history, assuming that they once had it. Men searched the temperate zone from 1843, when Montagne described *Monilia sitophila*, for half a century or more, yet the haplont described by Montagne remained a well-known nuisance without ascospores. Then I found it, for it was easily visible, in bagasse piles in Louisiana, and its ascosporic form, *Neurospora*, is common in the dirt and trash piles of Texas. *Neurospora* was found in several parts of the world about the same time, but

even though the ascosporic *Neurospora* is well known in the laboratory today, I do not know of its appearances in the bakeries of the North. In other words, the haplont seen by Montagne in Strasburg is a successful pest of the temperate zone, where its fellow has shown no evidence of ability to thrive. A geneticist quite acquainted with *Neurospora* assured me that the ascospores made it possible for the mold to survive the winter and other unfavorable conditions. Then I called his attention to the fact that ascospores had never been found in nature in whole regions where the haplont is well known.

These illustrations are presented to throw light upon a situation such as exists in *Penicillium* and *Aspergillus*, genera held together by quite characteristic conidia-producing structures, but which, in some series, maintain ascospore-producing species, while other series, often far more common as organisms in the field, show no trace of sex. Both series obviously belong in the same genus. To segregate either under another name would merely confuse the literature to no useful purpose. We must accordingly find methods to describe them both. In the controversy over *Aspergillus*, I searched the historic source badly dried herbarium specimens -through a few thousand exsiccati, using a good hand lens. There remained no doubt in my mind that yellow perithecia were certainly present in the mycelium of the *Aspergillus* of Micheli (1729), as well as of Link (1809).

If anyone looked seriously at specimens, there was no record of any such observation until DeBary (1854) made public his belief that these genera were upon the same mycelium. About all Link did was to take the yellow fruit bodies which Wiggers had called *Mucor herbariorum* (1780) and find that they contained asci, hence were not *Mucor* as Wiggers supposed, but the excuse for his new genus *Eurotium*. We followed Fischer (1897) in amending the generic characterization of *Aspergillus*, already in common use for conidial heads, by merely adding the completing observation that asci were produced in some sections of the group.

Have I made my point clear? The needs of academic mold studies, as well the requirements of industry, justify a systematic effort to establish a nomenclature for molds that we meet constantly and which present no trace of a perfect form. If or when perfect forms appear, questions of nomenclature can be settled, but one point is certain, a "perfect" strain in a well-known imperfect series is entitled to appear only as completion of the species description, not as a new name replacing the generic name already widely known. A species whose perfect form belongs in a well-established generic relationship can, of course, be transferred as a single unit without affecting other imperfect species. For example, *Paecilomyces* of Bainier (1907) is known wherever molds are collected. The ascosporic organism rarely found and vaguely designated as *Byssochlamys fulva* (Olliver and Smith, 1933) hardly justifies transfer of all of the well-known strains to *Byssochlamys*, even though the conidial forms in this species and in *Paecilomyces* appear to be closely related. In addition, it is by no means certain that the ascosporic strain described really belonged in Westling's genus *Byssochlamys* (1909). Until further study clarifies the situation, *Paecilomyces* is the better name, even for the ascosporic strain.

If rigorous morphological examination has been made the basis of separation

in the conidial forms, we have found that the ascosporic strains subsequently discovered are following nearly the same line of separation. In other words, there seem to be natural lines of separation which include both imperfect strains and perfect ones when found.

I have said little so far as to "what is a species among these molds?"

To the bibliographer, a species is a name reinforced by a technical characterization adequate to insure correct identification of certain organisms which properly belong together. Please note that this definition does not specify the exact description of some single unit designated as type and so preserved. I am deeply interested in preserving that type as a living culture if possible, or as a herbarium specimen, if such a specimen can be developed to aid in establishing identity, but its value is so often negligible that one is not certain, only hopeful.

Nomenclature that cannot be used by competent workers to identify fresh materials is a waste of ink. Microorganisms are not produced as machine-tooled structures. Descriptions which specify morphological identity to the fraction of a micron are misleading. Spores cannot be properly reported as showing diameters uniform to the tenth of a micron. Some investigators count and measure 100 spores, then analyze their figures statistically. Anyone who has examined hundreds of chains of spores and compared them as they stood in the chain cannot accept any such mathematical result as of serious value. Ranges of variation are helpful.

The sterigma, or fertile cell, aids in separating certain groups by its morphology. In the Biverticillata-Symmetrica section of *Penicillium*, the cells are mostly smaller in diameter, and each cell tapers to a long narrow tube. The cells of another series have shorter, abruptly constricted tubes, while the coarser series of blue-green forms have short broad tubes. The sterigmata, while showing the general character of the whole group, often show details that are significantly correlated to closely related series rather than to that strain alone.

The taxonomist is a morphologist. If he is also a microbiologist, he must use a microscope. If his organisms are really small, the best apochromatic objectives should be regularly at hand and used. I know places where using any oil immersion objective is a unique circumstance. There is a limit, however. I read a paper the other day in which the worker reported that the only dependable character separating his two species appeared to be the *length of the chromosomes*. I am afraid that the ordinary working mycological laboratories will be compelled to dump those organisms together and perhaps hyphenate their species' names.

Seriously, however, if the worker undertakes a research project using one of those two species, he must go the whole way if he is to settle moot questions. Research results which fail to establish, in advance, the identity of the organisms used should not be given publication space. Mere receipt of a tube with a name on it may help but cannot absolve the research man from the necessity of really knowing his organism. I have furnished a named culture to a man and, later, growing suspicious of his workmanship, have asked for a return transfer which proved that he was using a different species under the name.

I have said elsewhere that *Penicillium glaucum*, as a synonymn for green mold, competes with *Aspergillus niger*, a synonymn for black mold for the ghost position as the most versatile, biochemically, of all microorganisms.

Search for dependable criteria in describing these molds necessitated the study of great numbers of strains in culture. Needless to say, the spore-producing organs received special attention.

One striking constrast was ignored by the early mycologists who studied mostly, if not entirely, haphazard material from their environment. Some fungi produce branching chains of spores. Others show no branching. The difference is fundamental. In the whole area to which *Penicillium* and *Aspergillus* belong, the spore-producing cell is apparently defined by its function by the more or less unsatisfactory term *sterigma*. It is essentially a cylindrical cell tapering to a characteristic tube at the distal end. The nucleus divides (one daughter nucleus migrates into the lengthening tube) and a segment is cut off by a wall to form the spore, which continues to grow and assumes the size, shape, and markings of the species. Its nucleus then "rests." There is no further division until germination occurs as the beginning of a new colony.

The sterigma (basal cell) nucleus divides again meanwhile, and the spore (conidium) forming process is repeated. The end cell of the chain is accordingly the old cell, and the cell directly attached to the sterigma is the newest cell, while there may be hundreds between them. In all of the spores of the chain, the nucleus is a daughter nucleus of the sterigma nucleus. Every member of the chain presumably bears the common inheritance of that sterigma.

In contrast to the unbranched chain, another very large series of genera presents the branched chain. *Cladosporium* will be taken to illustrate the contrast. The terminal cell of the conidiophore, or fertile hypha, which may or may not show differentiation, puts out a bud—a bubble blown through a delicate opening in its wall. The bud swells, and one or many nuclei follow the cytoplasm into the bud, which assumes the pattern of the genus. Other buds may arise in the same way to form a cluster. The distal ends of such buds repeat the process which, in turn, is repeated over and over to produce a more or less characteristic mass, the newest cells forming the outside of the mass. Snyder and Hansen have described the process very fully. Even long chains may appear in this process and, as in the case of *Neurospora* (*Monilia sitophila*), branching may develop at any point.

I am quite aware that this whole discussion is brushed away by Shear as of no significance, but it must be noted that Shear was scarcely a cultural mycologist. He had some test tubes, but his room was full of dried materials which played no part in the industrial developments of today.

The two types of spore chain characterize separate groups of genera. The mutant of *Aspergillus niger* described by the Yuills (1938), if valid as a genus, would suggest possibilities of origin but does not appear to us significant. In the mutant described by them as *Cladosarum*, the nuclear story at the base of the chain is reversed. The resting nucleus remains in the basal cell, while the active nucleus migrates. The chain shows fantastic groups of cells more or less suggesting the basal cell, but always with the new ones at the tip, and quiescent cells in the chain.

Such mutants have been encountered often enough to justify this interpretation. They have not been reported in nature, probably because their propagative cells are not well adapted to distribution. Such strains are comparable to zoological monsters born only to disappear.

Another variation, probably also a mutant, shows little microscopic change in the sterigma itself, but the conidia appear double or more than double the size usual in *Aspergillus niger*. Raper and Fennell (1953) have demonstrated the presence of several nuclei in the sterigmata and conidia of *A. fonsecaeus*. They give no clue, however, as to how this multinucleate spored form might have originated.

The examination of these evanescent fungi may best begin with the actual conidium and its relation to the spore-producing cell (for this examination a high magnification, usually some type of oil immersion objective is necessary).

The next step calls for the type of aggregation of the spore-producing cells into some sort of fructification. The same preparation used in examining the spore-producing cell commonly furnishes the information as to the supporting structures. Once having the structural concept in mind, it is imperative to make free use of a dry objective in the examination of the margin or any portion of the colony which permits a study of the developing-fruiting apparatus with undisturbed spore masses (the worker is assumed to have ample reserves of slant tubes and petri dish cultures). Such observation requires removal of the petri dish cover and focusing the low-power objective upon undisturbed areas of the growing colony, both in full fruiting phase and in areas in which details of origin and development can be followed. I well remember the look on Thaxter's face when I uncovered a growing colony, put it under a microscope, and asked him to look.

The low-power examination rests for its adequacy of interpretation upon prior detailed knowledge, hence it is mentioned after rather than before the high magnification which furnishes the background for interpreting the habit picture.

By this time, in the examination, enough information is usually at hand for presumptive recognition of the genus, as covered in standard manuals.

In the borderland between *Penicillium* and *Aspergillus*, separation is not quite arbitrary because specialization for spore production in *Aspergillus* can ordinarily be traced back to a basal cell at the foot of the conidiophore. This "foot cell" increases in size and shows differentiation by thickened walls, while the conidiophore arises as a more or less perpendicular branch from its side wall.

Conidiophore

The spore-producing branch (stalk or conidiophore) may at first differ little in structure from a vegetative hypha. The typical habit of all these molds, however, is to project their conidium-producing structure into the air above the colony, so that the spores may be distributed freely. Reinforcement of the stalk wall is accordingly a response to strain, but the nature of that response

is commonly so distinctive that it needs to be determined under the best objectives. The simplest form of reinforcement from the observer's standpoint is the production, inside of the usually chitinous and very thin primary wall, of a comparatively thick secondary layer in which no structure is visible. That such walls may be permeable is frequently indicated in the undisturbed colony by droplets of transpired fluid variously scattered along the stalk. Such a liquid may be colored or colorless. Droplets may coalesce and flow away, or they may dry down to leave irregular patches of precipitate adherent to but in no way a part of the wall.

Again the thickenings may be regularly perforated at first, and later closed by new layers of wall within. Such thin points in wall-thickening clearly represent protoplasmic points of contact through the thin primary wall to the air. As the colony ages, such walls appear pitted or, if the pits are very numerous, they may appear to produce a rough surface. The primary wall may remain or peel off, so that we see only the secondary or reinforcement wall. Again, the pits filled with fluid may dry, to leave granular precipitate filling the pits and adding to the rough appearance. The important point is that ornamentation, no matter what its apparent form, is within the original or primary cell wall, with the few exceptions of droplets dried down.

Ornamentation

We must not forget that all of the structures that we see are cells, more or less transformed perhaps, but still cellular material. Ornamentation, whatever its form, must be accounted for as a result of activities inside the cell. If there are exceptions, they must be proved, not merely attached in an artist's drawing. This necessity was nicely illustrated when I followed Linossier (1891), soaked the richly ornamented *Aspergillus niger* spores in hot water, which removed their gorgeous black bars, and left thick, shining, colorless secondary walls loosely surrounded by their colorless primary walls.

We need, today, a series of papers on the actual nature of ornamentation in whole groups of species. Such a series would, I predict, clear away much confusion that covers spore descriptions as we find them today. It would require continuous study of the cells in all of these spore-producing organs, with the best microscopic aids available. There are literally hundreds of drawings of these cells as seen under low magnification that may satisfy a vague imagination, but such drawings furnish no clue to actual structure.

Spore Masses

In *Penicillium*, the cluster of spore-producing cells on various types of subtending branchlets got its name, *penicillus*, from the old writing brush of the Romans—hence the name *Penicillium*, applied by Link (1809) to the genus. Viewed in low magnification without comparative study, the penicillus was simply described as a brush. Painstaking comparative study establishes groups in which basically consistent branching systems throw series of related species together. The sterigmatic cells fall into several groups which aid in recognition of species. Definite clustering of supporting branches called *metulae* (by Westling, 1911), with ends more or less swollen, are characteristic

in sections of the group. No hard and fast penicillus structure can be safely depended upon, but general types are readily discerned.

Chains of spores may adhere into columnar masses based upon closely packed sterigmata or, at the other extreme, they may diverge so that no two chains are even parallel. The extremes are useful in sharp diagnosis. Less consistent forms are less helpful. Range in sizes of spores as commonly seen is important. Extremes in size are often desirable as contributory information. Exact figures to the fraction of a micron are misleading.

In *Aspergillus*, the sterigmatic cells are arranged in a compact layer or double layer on the vesicle, a swollen or flattened apex of the stalk, or conidiophore. Again, the unit is the final sterigma and its chain of conidia, but the structure of the head contributes the paramount species character for identification.

Colony

Colony morphology is significant. Critical comparison of colony habit must not be overlooked. Upon a standardized nutrient medium growth habit is commonly dependable. One colony may spread rapidly through the whole area presented. The submerged mycelium may outgrow the aerial hyphae, or *vice versa*. Again, colonies may be compact and consistently restricted in habit. Each conidiophore may rise from a submerged hypha, like culms of wheat, or all or some of them may branch from hyphae in the air above the surface of the nutrient. Such aerial hyphae may form an evenly surfaced floccose layer, or they may be composed of long trailing, interlocking hyphae, or of hyphae packed into bundles, or ropes, usually trailing over the surfaces. Again, upon standardized media the observation will be consistent.

This observation is repeated here because I have seen laboratories in great industries where the person in charge has discarded the nutrient used in book descriptions and ordered the substitution of different formulae. I have consistently advised such folk to sell their manual and write another if they prefer, but not to expect to check an unknown mold grown upon someone's whim as a choice of substratum against a description written from standardized culture.

Color

Color has been an element in mold description clear back to Micheli (1729). It is the first seen and most striking feature of many molds in nature. Discoloration of the substratum has been frequently reported. When subjected to cultural analysis, color is so frequently found to be related to the composition of the nutrient substance that observations unrelated to such known nutrients may be misleading. A Japanese friend described a member of the *Aspergillus flavus-oryzae* series as bright green in the fumes of glacial acetic acid but turning brown in the presence of ammonia. This change, however, was reversible back to green if the colony was again subjected to the acid. He sent the culture to me, and it acted as reported. I examined at least 100 strains of that series, however, and they all gave the same reaction. The shades in that series were shown to vary from intense greens, under acid conditions through many shades, to brown in old alkaline plate cultures. Again, red to yellow colors in the nutrient medium are conspicuous features of particular

species, but experiments commonly relate each color to the action of the species upon components of the nutrient. Hence, with the same strain, the color may differ with the formula used in preparing the substratum. Not infrequently, the color in the medium alters with the age of the colony. Both the nature of the medium and the physiological age of the colony accordingly need to accompany the report on the color.

Reaction

Critical comparison of culture formulae and reaction changes as a consequence of mold growth show that, in a single culture vessel, there may be unaffected media at approximate neutrality, zones of acidity as low as pH 4.0 and progressive alkalinizations up to pH 7.5 in the center of a colony. Such progressive changes of reaction are definitely reflected in colony color and in the colors or consistency of the substratum. If the pH relation is ignored and a different carbohydrate used in the nutrient, a quite different aspect of the fungus colony is reported. Recognizing fully that the mycological laboratory cannot analyze these complex relations culture by culture, one must again emphasize that, for descriptive study of such organisms, rigorous standardization of the medium permits comparative study capable of repetition in different laboratories. Almost limitless variation is possible but not practical for diagnostic purposes.

Discussion

I have sought to establish the necessity of describing species among these asexual molds in such a way as to enable both academic and industrial laboratories to understand each other and to recognize the strains used as representing definite species under correct Latin names. I am quite aware that no one has ever been able to describe certain mutants of great commercial value. The unbalanced character of such mutants, in that one activity has been enormously developed at the reduction if not actual elimination of others, puts them apparently in the group of abnormal or "monster" organisms which may be preserved in protected culture but would die out and disappear in normal environments. Such organisms are not entitled to places in any taxonomic program.

Variability is a well-recognized character of asexual organisms. Such variability in morphology and activity, however, ordinarily follows fairly well-marked lines. Occasionally, we are convinced that new species are developed which, by their vigor and aggressive characters, become established in nature and hence accepted in nomenclature.

Diagnostic description as a basis for nomenclature calls for rather complete data, itemized somewhat as follows: (1) colonies upon a designated culture medium; (2) colony size as attained in a definite period at stated temperature; (3) character of the marginal area of the colony; (4) distribution, appearance, and color of sporulating area, and variation of color within different colony areas; (5) color of reverse of colony, with or without coloration of medium; (6) exudate (transpired drops), if any; abundance, color; (7) texture of the mycelial wall and any characteristic markings; (8) conidiophores: method of

formation, length, diameter, arrangement with reference to each other; (9) conidiophore walls: character, color, markings; (10) branches, if any, and metulae: dimensions, markings, if present; (11) penicillus or conidial head: general structure, measurements with undisturbed spore mass; (12) sterigmata: verticillate arrangement, measurements, pattern; (13) conidia: variation in shape and size, ornamentation, color; (14) sclerotia or other specialized structures; and (15) accessory information: origin or activity in nature, if known; geographical or climatic data; special observations upon other media.

Aggregate Species

This question naturally arises: have the preceding paragraphs created the idea of a clear-cut concept of species as described? Critically compared, many strains among omnipresent molds encountered present the general picture of some species created by the descriptive procedures already outlined, yet retain individual aspects which separate them fairly readily when grown side by side. Found separately, no satisfactory description of shades of color, details of habit, or even spore formation has made separation possible. In fact, as strains accumulate, variability within a general concept is inescapably recognized, with the further observation that no sharp lines between strains remain. Since a particular strain might be arbitrarily selected as type upon the basis of priority, or otherwise, the thoughtful mycologist (conscious of the variability encountered) will tend to regard the many strains together as representing merely different facets of the same species. For convenience, such an array of intergrading strains can be appropriately regarded as constituting an *aggregate species.*

Penicillium expansum may be used for illustration. The fasciculate arrangement of the conidiophores, the characteristic odor, and the ability to rot apples seems to be common to all strains. The shades of green in the outer zones, the prominence of fasciculation, and the colors in the substratum vary appreciably. Looking at hundreds of them, however, leaves little room for doubt as to their proper placement. No one knows which strain Link found in Berlin upon "fruit." I hunted the fruit stalls of Berlin and found many rotting *Mespilus* (the native *Malaceous* genus) showing the characteristic coremia with well-marked green heads. Transfers from similar structures give the molds as we see them in our collections. In our manuals, we could find no satisfactory escape from the discussion of such groups of strains as aggregate species. The same reasoning applies over and over again among the *Penicillia* and the *Aspergilli*. If we have been able to guide the user of the manuals to aggregates of strains among which differences are quantitative, not qualitative, and not associated with clear-cut differences in details of morphological structure, we have accomplished our purpose; *i.e.*, we have opened the literature of the aggregate to the worker.

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SPONTANEOUS AND INDUCED VARIATION IN SELECTED STOCKS OF THE *PENICILLIUM CHRYSOGENUM* SERIES

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Our work on *Penicillium* began under government auspices during World War II, when our sole objective was to develop strains of the mold capable of producing higher yields of the then new "miracle drug," penicillin. Later, however, our program was broadened to include diverse fundamental studies on variation phenomena in selected stocks of the *Penicillium chrysogenum* series. From this point on, the relative ability of a variant to produce the antibiotic became only one of many characters to engage our attention. Nevertheless, there have emerged from our general program a series of superior strains, well known in industrial circles. The first notable strain in the Wisconsin Series, Q-176, was released from our campus late in 1945.

It is not our purpose today to lay special stress on these outstanding strains. Neither shall we discuss in detail any of the various individual facets in our program of fundamental studies. Rather, it is intended to point out something of the range of departures from the wild-type ancestors encountered among the nearly 30,000 strains which have been dealt with in the course of our work and to call attention to certain remarkable variation phenomena occurring in the group of organisms with which we have been concerned.

A variety of isolates of *P. notatum* Westling and *P. chrysogenum* Thom passed through our hands and formed the basis for operations in our laboratory during the initial stages in our program, but the bulk of our strains trace their origin from a single ancestral race the famous Northern Regional Research Laboratory isolate of *P. chrysogenum* known as NRRL 1951. Before this strain was available, however, our chief attentions centered on the *P. notatum* strain, NRRL 832.

The variants which we have obtained arose in a number of different ways: through spontaneous change; through various individual mutagenic treatments; and through diverse combinations of repeated treatments and selections over long series of generations.

Spontaneous variation is most interesting and, of course, fundamental. In the stocks with which we have worked, miscellaneous spontaneous variants have appeared in the usual manner, namely, arising as sectors or spot overgrowths on colonies grown on agar, or showing up as distinctive colonies in a population grown from individually isolated spores of the parental race. In addition, variants which do not possess any distinctive cultural traits, but which nevertheless differ from their parent in their physiology, may occur. Special tests are, of course, necessary to detect these variants.

The effectiveness of certain mutagenic agents in increasing the incidence of variations is now well established for a considerable range of microorganisms. One of the most effective agents, and the one which has been most extensively employed in our laboratory, is ultraviolet radiation. Certain chemicals tried

out in our laboratory have also been found to be satisfactory. The most effective of these agents, and the one used more than any of the others in our studies, has been the nitrogen-mustard, methyl-bis(β-chloroethyl)amine. Acenaphthene, camphor, chloral hydrate, *etc.* have been employed, however, to some extent. One consideration of great interest and significance is that we apparently can secure, through irradiation and chemical treatments, types of variants which either do not occur naturally at all or which appear so rarely that we have not yet detected any of them.

Before turning to a consideration of the range of characters involved in the variations which we have encountered, it seems desirable to comment briefly on what we refer to as the "Wisconsin Series" or "Wisconsin Family" of *P chrysogenum* strains (FIGURE 1). It is especially important to point out that this designation, as we have used it, does not by any means comprehend all of the strains which have emerged from our studies. Rather, it is applied only to what we might term our elite stock. As we define it, the "Wisconsin Series" begins with strain Q-176 and includes all of the derivatives of this strain which have been secured in nine years of selective work in our laboratories. The key strains in this series have all been selected with due attention to their ability to produce penicillin. Although, for obvious reasons, this particular group of strains is the one best known outside of our own laboratory, most members of the "Wisconsin Family" are actually poorly adapted to some of the types of fundamental studies which we have desired to complete. We have accordingly

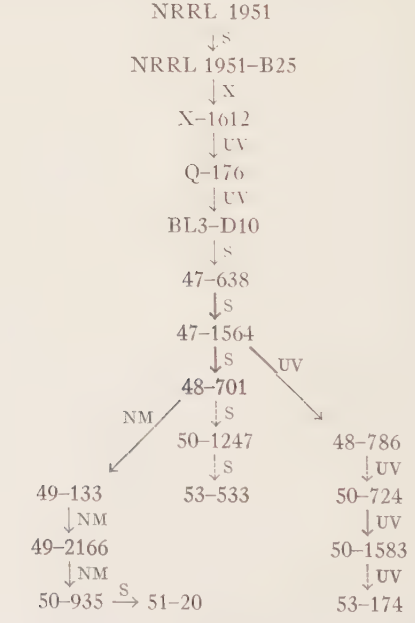

FIGURE 1. Geneology of the Wisconsin Family of strains of *Penicillium chrysogenum*. (S, selection without mutagenic treatment; X, selection following X irradiation; UV, selection following ultraviolet irradiation; NM, selection following nitrogen mustard treatment; solid arrow, no intervening generations; broken arrow, one or more intervening generations.)

used other stocks, especially the ancestral form NRRL 1951, for material more suited to certain of our needs.

Turning now to a description of some of the departures from the wild-type ancestors, we may begin by considering the color variants. These variants, perhaps, impress one most at first, and they are common in all of our stocks. Some of these strains differ from the parent cultures essentially only in the color of their spores; but the color of the mycelium may be involved in other cases and, not infrequently, the colony morphology is modified as well. Color variants are especially prominent among the survivors of mutagenic treatment, including ultraviolet irradiation, where an almost complete spectral range from light blue, through green, to yellow, orange, and reddish brown may be encountered. Colorless variants are also quite common.

The colorless variants themselves form an interesting group. These variants can be divided into two categories: (1) the pleomorphic, or nonsporulating albinos and, (2) the white-spored, or true albino forms. While both of these types of colorless variants occur throughout the "Wisconsin Series" of strains, with the true albinos the most common, the only intensive study of albinos in our laboratory was made a number of years ago by Doctor O. H. Calvert, who used, principally, *P. notatum*, strain NRRL 832, as the parental stock.

Altogether, Calvert assembled a collection of 100 albinos of diverse origins, 50 of which he studied carefully. No two of these albinos appeared to be exactly alike when grown on three differential media, although a few were very similar. Some of the albinos arose spontaneously; others following ultraviolet irradiation, treatment with chemicals, *etc.* Among the true albinos, marked differences in growth rate, production of exudate, and production of yellow pigment were observed. Differences in colony topography were also recorded. The pleomorphic strains differed in these same features. Most of them produced a fluffy, white mycelium, but a few had a mycelium which was pale yellow or pink.

P. notatum strain NRRL 832, the parental form for most of Calvert's albinos, is shown in PLATE 1, FIGURE 2. It is seen here as it appears on the three differential media* which Calvert used. In FIGURE 3, we see a sporulating albino of spontaneous origin. The colonies are of the same age as those of the parental strain. FIGURES 4 and 5 show two very similar pleomorphic variants. The variant represented in FIGURE 4 originated through an irradiation treatment, while the strain represented in FIGURE 5 was of spontaneous origin.

The profound effect which the medium itself may have on the color, rate of growth, degree of sporulation, gross colony morphology, and pigment production is readily observed by growing strains on several differential media, as was done in Calvert's study. The response of a strain may be attributable more or less directly to the presence or absence of one or more constituents, to the pH, or to some other characteristic of the medium on which it is grown. For strains such as NRRL 1951, Q-176, and Wisconsin 49-133, a medium containing only mineral salts and sucrose is more or less satisfactory for growth. That

* The following three media were employed: (1) honey-peptone agar (6 per cent honey, 1 per cent Difco bacto-peptone, 2 per cent agar); (2) Czapek Dox agar (see Raper and Thom, *Manual of the Penicillia*, Williams and Wilkins, 1949, p. 64); (3) Peoria Nutrient Agar No. 1 (3 gm. cerelose, 5 gm. bactopeptone, 3 gm. yeast extract, 15 gm. agar, to 1000 ml. with distilled water).

is to say, there are no other growth requirements, although a better growth response may be elicited by adding other substances to the medium. In spite of the fact that all of these strains evolve spontaneous variants which differ from their parent in a variety of characters, it appears that very few, if any, of the spontaneously occurring variants are of the type one would designate as "biochemical variants." On the other hand, biochemical variants are always found among the colonies formed from spores surviving ultraviolet or nitrogen-mustard treatment.

Before leaving the matter of special nutritional requirements, attention should be directed to another very interesting type of variant: one characterized by a partial requirement for the mutagenic chemical employed in its induction. A colony of Q-176 which has been surrounded by a barrier of acenaphthene crystals is shown in PLATE 2, FIGURE 6. The first response of the colony subjected to this kind of treatment is a marked reduction in rate of growth and amount of sporulation. Within an incubation period of four weeks or less, however, the colony almost invariably produces one or more rapidly-growing, heavily-sporulating sectors which grow out over and through the acenaphthene barrier. When hyphal-tip transfers are made from these sectors onto honey-peptone medium, the colonies produced grow much more slowly and sporulate less abundantly than the parent. If they are transferred to honey-peptone agar containing an excess of acenaphthene, colony growth and the production of conidia are almost normal.

This curious behavior is illustrated in FIGURE 7, which shows two of the acenaphthene-requiring strains growing in the absence and in the presence of the hydrocarbon. The acenaphthene requirement is not complete. These variants, however, are extremely stable in regard to the requirement. Doctor Roy Curtis, who has recently completed a study of acenaphthene-requiring strains, has found that ease in inducing this type of variant appears to decline as we progress through the Wisconsin Series; that acenaphthene cannot serve as a sole source of carbon for these variants; and that, strangely enough, in corn-steep shake flask fermentations, the acenaphthene-requiring strains produce less mycelium but more penicillin in the presence of the hydrocarbon. Production of the antibiotic, however, does not reach the level of that of the parent strain from which the variant was derived.

As has already been suggested, variant strains often exhibit a change in gross colony morphology. Indeed, this change is one of the commonest types of modifications encountered. The variations in this category are almost endless, ranging from slight to profound departures from the parental form. Raised colonies showing a lanose texture, slow-growing colonies appearing almost sclerotic, wet-appearing colonies, and thin colonies having irregular, arachnoid margins are among the numerous kinds which have been observed. Many of these strains have little resemblance to colonies of the wild ancestral type.

We may next consider variations at the microscopic level and, in this connection, we particularly wish to report some observations made in our laboratories by Doctor T. H. Campbell, Doctor Bruce W. Churchill, and others, concerning the fruiting structure and vegetative hyphae in strain Q-176. Two

conidiophores of strain NRRL 1951, the wild-type ancestor of the entire "Wisconsin Series," are shown in FIGURE 8. In contrast, representative conidiophores of strain Q-176 may be seen in FIGURE 9. In addition to a general reduction in the size of the fruiting structure, great irregularity is observed in some of the penicilli. In the more extreme cases, the normal orientation of parts is lost, sterigmata of very unusual shape may appear (as in L), and, as the ultimate in reduction, the conidiophore bears a single sterigma (as in I). In general, as we progress through the Wisconsin Series of strains, farther and farther from the wild-type ancestor, and into the stocks giving high penicillin yields, the greater the proportion of fruiting structures which depart strongly from the type encountered in strain NRRL 1951. The most drastic departures may be seen in some badly-damaged strains surviving mutagenic treatment. In many strains having extensive aerial mycelium, the conidiophores arise here and there, apparently at random, from various aerial hyphae.

Irregularities of vegetative hyphae are equally pronounced throughout the Wisconsin Series. Such irregularities are well illustrated in strain Q-176. Two colonies representing the extremes in colony types characteristic of this strain are shown in PLATE 3, FIGURES 11 and 15. The latter is the remarkable A-type colony, which produces no spores although what appear to be abortive conidiophores may sometimes be found. A portion of the mycelium of an A-type colony is shown in FIGURE 10. While some of the hyphal segments are normal in appearance, others are greatly distorted and enlarged, forming distinctive globose or barrel-shaped giant cells. The giant cells and certain other mycelial peculiarities are especially characteristic of the A-type colony, but they are found in some amount in all of the colony types represented in strain Q-176.

While considering morphological variability, mention should be made also of the variation in size of spores in a single strain. Spores varying considerably in size and shape are of common occurrence in all of the strains of the Wisconsin Series.

Another feature of the variants with which we have been concerned is the range in their capacity to produce penicillin. Because this feature involves the best-known phase of our work, we can perhaps dispose of it quickly here. Suffice it to say that it has been possible to develop variants giving yields of the antibiotic up to 20 times as great as those obtainable from the wild-type ancestor.

Penicillin yields of various strains in the Wisconsin Series of *P. chrysogenum* are shown in TABLE 1. The data are from a shake flask test. Higher yields can be secured from all of these strains under more favorable fermentation conditions.

Pigment production is another character in which strains exhibit marked differences. Such production, again, is a character of considerable interest and importance in relation to the commercial production of the drug. The specific epithet "chrysogenum," in the name *Penicillium chrysogenum*, refers to the excretion of a golden-yellow pigment which is listed as one of the characteristic traits of the species. Strain Q-176 and its progenitors produce such pigment in large amounts. In the course of our studies, however, we have encountered numerous variants which did not produce any pigment at all, and others

TABLE 1

SHAKE FLASK YIELDS BY SELECTED STRAINS OF THE WISCONSIN SERIES OF *P. chrysogenum*, RUN NO. 286

Strain	No. of Flask	O.U./ml. Av. 6 days	O.U./ml. Av. 7 days	O.U./ml. Range 7 days
Q-176	8	293	435	255–600
47–638	8	307	486	415–540
48–701	8	507	586	480–620
49–133	8	872	1519	1380–1780
50–935	8	996	1741	480–2200
50–1247	8	710	1097	980–1310
50–1583	8	543	1098	1000–1350
51–20	8	1308	1934	1530–2550

Conditions: Corn steep-lactose medium, with 0.25% betaphenylethylamine; reciprocating shaker, 4 in stroke, 92 cycles/min.; T, 24–25° C.

which varied greatly in their capacity to produce pigment. An additional item of interest is the occurrence of variants which produce a pigment, or pigments, other than the characteristic golden-yellow types. For example, strains NRRL 1951 and Wisconsin Q-176 are strong producers of yellow pigment. Strain 47-1564 produces as much penicillin as Q-176, but it does not secrete any pigment into the fermentation medium. Further, strain 49-2105 is remarkable in that it produces a dark reddish-brown pigment.

One of the most interesting and baffling aspects of spontaneous variation in the Wisconsin Family of strains of *P. chrysogenum* is involved in what we have called the "population pattern phenomenon." This aspect has been most carefully studied in the case of strain Q-176, but it appears to occur widely throughout the Wisconsin Family of strains.

If a large population of single-spore colonies of Q-176 is grown on honey-peptone agar in such a manner that each individual colony is well separated from every other colony, the striking character of the population becomes evident after about seven days incubation.

Populations of strain Q-176 so set up exhibit great heterogeneity, but the remarkable thing is that we are able to catalog almost every colony under one of five major types. We have designated these types as the U-, D-, C-, B-, and A-types, and we find that, in any sizable population, there is always a fairly definite percentage of colonies of each kind. This fact suggests that some orderly mechanism is operating here, and it also enables us to define the strain, in part, on the basis of its population pattern. A typical Q-176 population pattern is shown in TABLE 2. Since U-type colonies predominate, we designate Q-176 as a U-type strain.

The five major types of colonies found in a Q-176 population are illustrated in PLATE 3, FIGURES 11-15. The U-, D-, C-, B-, and A-types, in that order, seem to represent a "sliding-downward series." That is, as we descend the series, we encounter progressively decreased sporulation accompanied by progressively increased abnormality of mycelium and reproductive structures. From the U-type through the B-type there is also a progressive reduction in the size of the colony in terms of radial spread. A-type colonies sometimes have as great a diameter as do the C-type colonies, but they produce no spores at all and show a curious flesh color.

TABLE 2
POPULATION PATTERN OF STRAIN WIS. Q-176

Colony type	%
U	64.8
D	20.2
C	8.5
B	2.6
A	3.7
All others	0.4
Total	100.2

In another sense, also, the succession of colony types represents a "sliding-downward series," for, as the series descends, a nearly complete dropping out of the "higher" types occurs in populations grown from spores. That is, conidia from a given type of colony yield predominantly colonies of that same kind, plus a definite proportion of all types lower in the scale, but rarely any colonies of a higher type. It has been found, however, that in Q-176 and also in some other races, a reversal of the sliding downward trend can be effected by allowing colonies to sector. Sectors arising on colonies "low" in the series are almost invariably of a "higher" type, and the tendency to sector grows progressively stronger as we move downward in the scale. A- or B-type colonies growing in isolation tend to sector early and abundantly. An A-type colony having a U-type sector is shown in PLATE 3, FIGURE 16.

There are a great many other facets to the population pattern phenomenon which we wish might be discussed here, for we believe that we may have uncovered, in the whole behavioristic pattern involved in the situation, something of fundamental interest.

Our authorities on the Penicillia regard strain NRRL 1951 as a very typical representative of the species *P. chrysogenum*. But already, as early as 1945, Raper and Alexander had secured from this basic stock spontaneous variants which, they stated, covered almost the entire gamut of cultural types seen in the *P. chrysogenum* series. They also reported, in certain of these strains, the occurrence of abnormal penicilli. Many of the variants which have emerged in our laboratories show similar but even more exaggerated departures from the wild-type ancestor. Certainly, among the descendants of strain NRRL 1951 which we have seen and worked with, there have been hundreds which diverge from the ancestral type in so many ways and so radically, in regard to some of the characters, as to have little left to suggest, at first examination, that they are really *P. chrysogenum*, or even members of the *P. chrysogenum* series.

Some enduring traits remain, however, in many of the otherwise highly modified forms. The ability of conidia to grow after long periods of dessication, which is a notable feature of the *P. chrysogenum* series, persists as a character in all of our strains which have been put to the test. Conidiophores and penicilli, however distorted, have remained smooth-walled in all cases where these structures have been examined. Another durable feature is the production of penicillin. In very few of our variants has the ability to produce the

antibiotic dropped to the zero point or even approached it. Any mycologist receiving as unknowns certain of the extreme variants from our Wisconsin Series of strains might be sorely perplexed by them until he did one simple thing: namely, ran a penicillin production test. The substantial or even enormous yields of the antibiotic which can be secured with many of these peculiar strains would soon give strong indication as to where they belonged. Nevertheless, it is possible to obtain strains producing either no penicillin at all or amounts less than have been obtained even from some other genera of fungi. Although such have been rare indeed in our laboratory, we have every reason to believe that, had we worked as diligently to select low producers as we have to develop strains giving high yields, we could have in our possession today a whole family of variants lacking the ability to produce the antibiotic.

The definition of *P. chrysogenum*, as given in Kenneth Raper and Charles Thom's *Manual of the Penicillia* is drawn along relatively broad lines. It allows a considerable range in many of the characters. Furthermore, the authors of this manual have pointed out that it is impossible to draw any sharp lines of separation within the *P. chrysogenum* series, owing to the prevalence of intergrading strains. Thom, in 1930, listed nine species as belonging to this group but Raper and Thom now recognize only four. They appear to have considered, but rejected, the possibility of regarding the entire group as one variable species. We do not propose to enter into the debate on what a species is. We believe that criteria for speciation will inevitably be different from one group of organisms to another, and we hold that the criteria are best set up by those who have had the broadest possible experience with the particular group.

We are not prepared to say how much weight should be assigned to laboratry variants in formulating concepts of species. How much of the variation which we have seen is duplicated in nature, we have no way of knowing. Undoubtedly our most extreme variants, if they did appear spontaneously, would have no chance of survival under natural conditions. Others would seem to have a fair chance of competing successfully. Knowing the origin of our strains, we confidently refer all of our NRRL 1951 derivatives to *P. chrysogenum*. If some of these had been isolated from nature, however, we wonder what disposition might have been made of them.

We shall be content to let the taxonomists draw their own conclusions from the facts and decide what, if any, the implications for speciation may be. We do believe that it is worthwhile to emphasize that, in nearly ten years of working with the descendants of a single isolate of *Penicillium chrysogenum*, the range and amount of variation which we have witnessed has far exceeded anything we could foresee when first we began work on our project.

PLATE 1

P. notatum, strain NRRL 832, and three albino variants derived from this strain growing on three differential media: left to right, honey peptone, Czapek's-Dox, and Peoria No. 1.

FIGURE 2. Colonies of *P. notatum*, strain NRRL 832.

FIGURE 3. Colonies of a sporulating albino of spontaneous origin.

FIGURE 4. Colonies of a pleomorphic variant obtained following ultraviolet treatment.

FIGURE 5. Colonies of a pleomorphic variant of spontaneous origin.

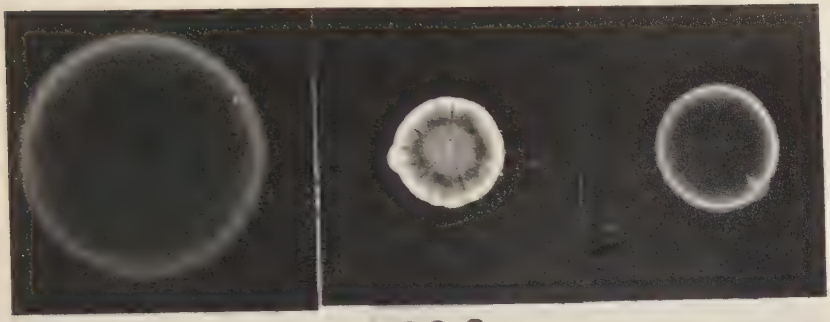

FIG. 2

FIG. 3

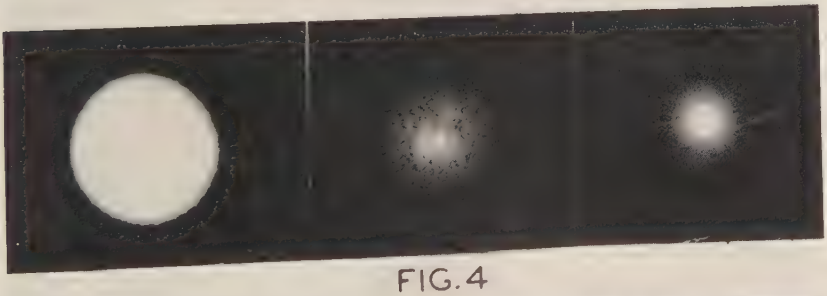

FIG. 4

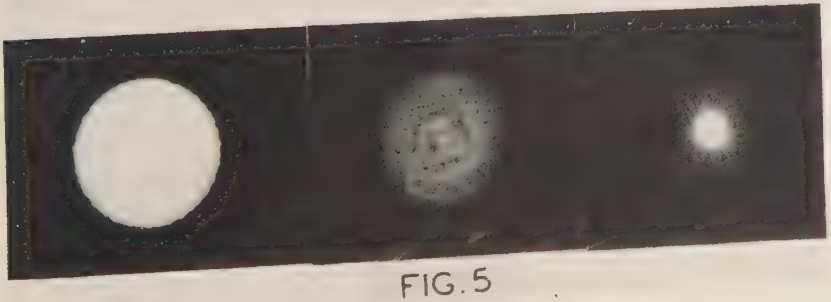

FIG. 5

PLATE 2

FIGURE 6. A young colony of *P. chrysogenum*, strain Wis. Q-176, surrounded by a barrier of acenaphthene crystals.

FIGURE 7. Colonies of two acenaphthene-requiring strains (Q-176-1 and Q-176-2) growing in the absence (above) and in the presence (below) of acenaphthene.

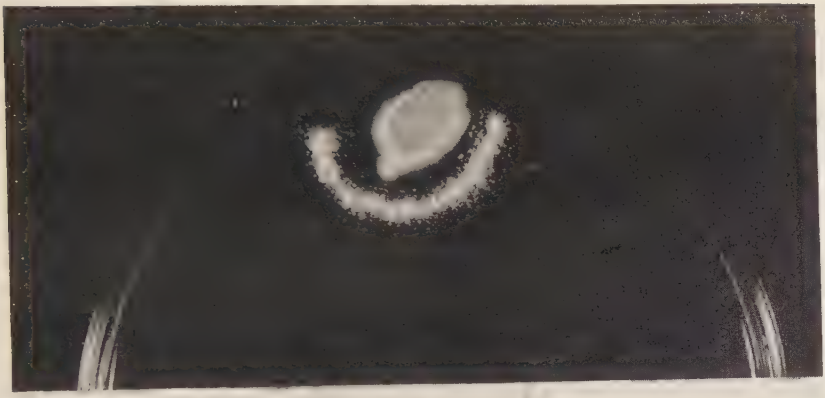

FIG. 6

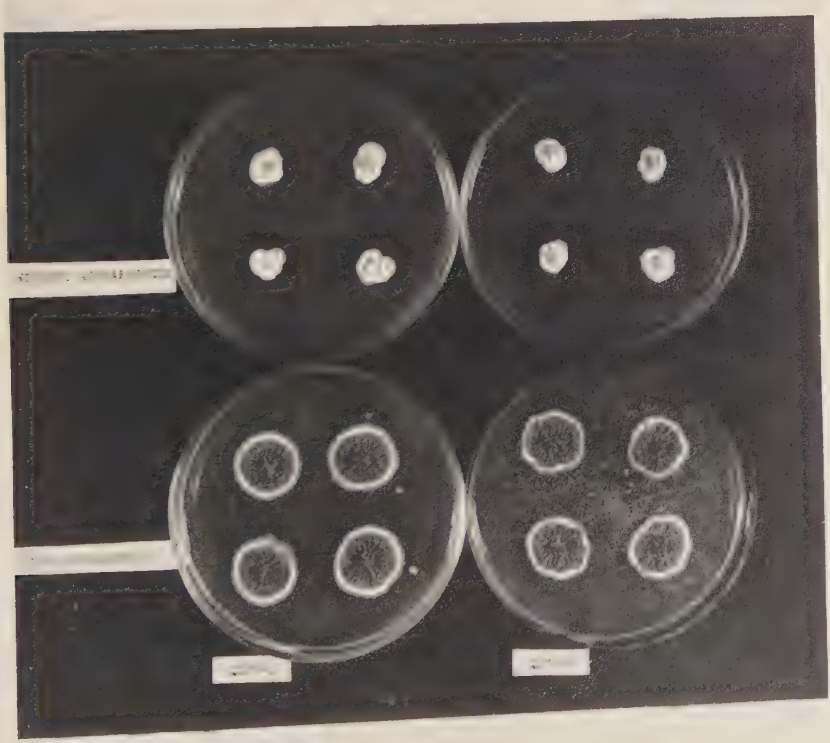

FIG. 7

FIGURE 8. Conidiophores of *P. chrysogenum*, strain NRRL 1951.
FIGURE 9. Conidiophores of *P. chrysogenum*, strain Wis. Q-176.

FIGURE 8

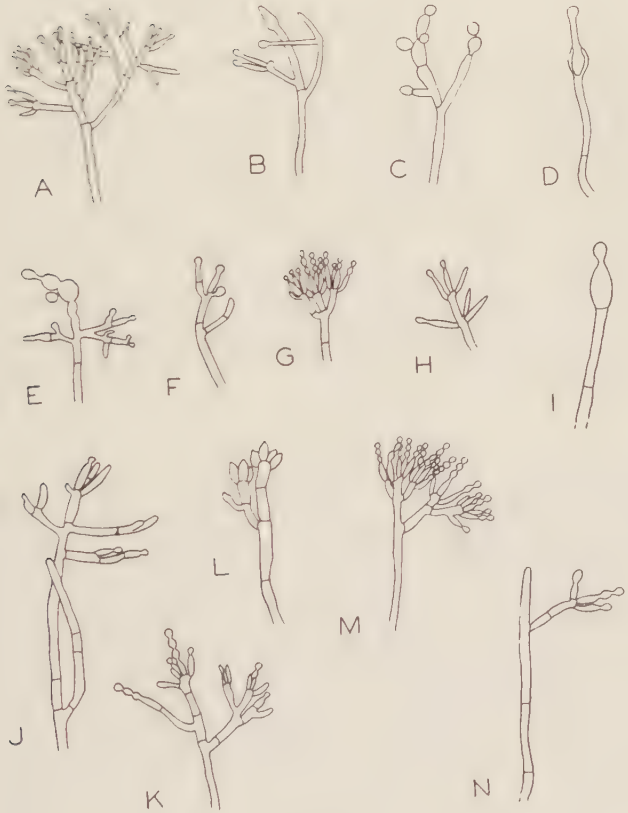

FIGURE 9

PLATE 3

FIGURE 10. Photomicrograph showing abnormalities of hyphae of an A-type colony of *P. chrysogenum*, strain Wis. Q-176.

FIGURES 11–15. The major colony types of strain Wis. Q-176 growing on honey-peptone agar (FIGURE 11, U-type; FIGURE 12, D-type; FIGURE 13, C-type; FIGURE 14, B-type; FIGURE 15, A-type).

FIGURE 16. An A-type colony of strain Wis. Q-176 with a U-type sector.

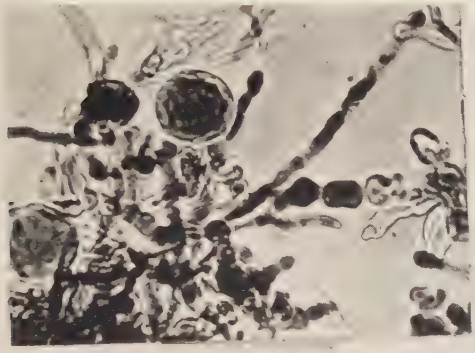

FIG. 10

FIG. 11 FIG. 12 FIG. 13

FIG. 14 FIG. 15 FIG. 16

EXPERIMENTAL CONTROL OF MORPHOGENESIS IN MICROORGANISMS

By Walter J. Nickerson

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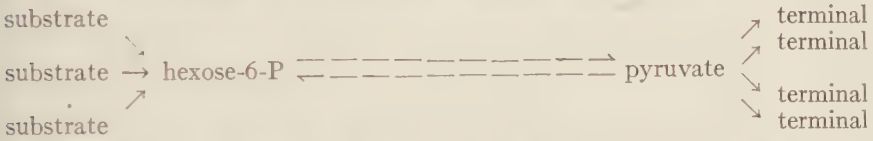
The environment in which the growth and development of microorganisms take place acts more or less directly to control expression of the morphogenetic potential innate in a microbial cell. This concept has been long appreciated, and numerous instances of experimental control over the form of microbial growth are recorded in the older literature.[1-4] In recent years, increased attention has been paid to the manner in which this control is exerted in an effort to learn something about the physiological bases of morphogenesis. Examples of such efforts include studies on the diffusible organizing factor for *Dictyostelium discoideum*;[5] demonstration of nutritional independence of temperature-dependent M $\rightleftharpoons$ Y transformation in *Blastomyces dermatitidis*[6, 7] and in *Paracoccidioides braziliensis*;[7] the influence of nutrition on the form of *Blastocladiella*;[8] and the importance of exogeneous carbohydrate metabolism for the reduction of disulfide groupings and consequent maintenance of the yeast state in *Candida albicans*.[9, 10] Likewise, increasing attention has been paid to the effect of specific environmental factors on the form of bacteria. The uncoupling of cellular division from growth, without impairment of growth, has been achieved by a variety of methods.[11, 12] Consequently, a means has been provided to study aspects of growth in the absence of cellular division and to probe a variety of microbial types for enzymatic reactions involved specifically in cellular division.[13-15]

Types of Metabolic Pathways Involved in Morphogenesis

It is well known that, in most microbial types, one may obtain a so-called "resting cell metabolism" by supplying only a metabolizable carbohydrate to washed cells. This resting cell metabolism comprises a transformation of substrate, to yield metabolic products, and energetically coupled polymerization of carbohydrate intermediates(s) to polysaccharide(s). It is likewise well known[16] that the latter reaction (so-called "assimilation") may be uncoupled from degradation pathways without impairment to these pathways. Against this background, the occurrence of growth in a carbohydrate metabolizing system (by addition of a nitrogen source) and the occurrence of division in cells that can grow in the absence of division may also be viewed as examples of the coupling of synthetic reactions (polymerizations, reactions leading to increased orientation) to degradative, exergonic reaction systems. Recently, a site of coupling of the division mechanism to oxidative processes has been demonstrated in certain yeasts to occur at a metallo-flavoprotein locus.[17] From these factual beginnings, one may advance the supposition that the metabolic reactions responsible for the syntheses involved in growth, division, and differentiation depend upon simple energetic reactions by coupling at relatively few points.

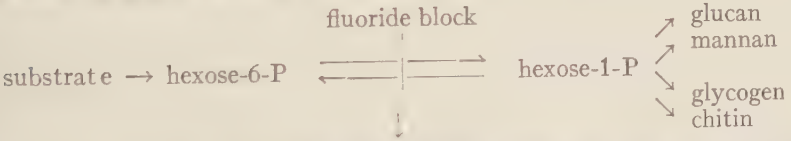
Umbreit,[18] in an analysis of the fundamental principles of the construction

and operation of metabolic pathways involved in substrate degradation, has emphasized that the term "pathway" might better be replaced by "highway." Oxidative and fermentative routes are characterized by long uncrossed routes that stretch link after link, from a metabolic exchange lying close to the substrate level, to a metabolic exchange lying close to the terminal product. One may thus visualize the route as a thruway into which specific substrates may be fed at interchanges, and from which specific destinations may be reached in short terminal steps. One important route might be depicted as follows:



This view is not, of course, speculative, but is a generalization of the vast factual information available on the mechanisms of enzymatic degradation.

No comparable mass of factual information is available for the synthetic routes leading to the terminals responsible for the form of a cell, but such information as is available indicates that a generalization, similar in outline to that for degradative through ways, may also hold for synthetic thruway. One direction of synthesis essential to the growth of yeasts (and, quite likely, with some variation to many other organisms) may be depicted as follows:



The *in vitro* synthesis of glycogen from glucose-1-phosphate has been intensively studied by the Coris and by other workers. It has been shown that the synthesis of glycogen results from the addition of glucose-1-phosphate molecules to a pre-existing polymer chain, the coordinated operation of two enzymes being necessary to obtain the characteristic linking and branching of glycogen. The *in vitro* synthesis of mannan, glucan, or chitin has not yet been realized, but studies[19, 20] on the hexose-1-phosphate reversal of fluoride inhibition of growth lend credence to the view that these polysaccharides also originate from hexose-1-phosphate esters, although the coordinated operation of three enzymes might be necessary to achieve the synthesis of a mannan with the requisite branching known to be characteristic of yeast mannan.

It seems worthwhile to emphasize that the relatively few enzymatic transactions involved in moving glucose to the polymer glucan result in the synthesis of a fabric that can be a "yeast" in outward appearance. If baker's yeast is successively extracted with acetone-alcohol, ether, and strong sodium hydroxide, all components of the cell except the glucan envelope are removed. In the light microscope, this residue may give the appearance of an undamaged yeast cell, but after a period of treatment with hot dilute acid the electron microscope may reveal the fibrillar nature[21] of this single molecular species, the

structural arrangement of which is known in some detail. Distinctions between "morphological" and "physiological-biochemical" characteristics of an organism are accordingly based on a dichotomy of thought that may result in a simplification that has some pragmatic value for the taxonomist, but has no basis in fact. The form of a microorganism is dependent upon enzymatic syntheses that are susceptible to environmental influence, as are the enzymatic degradations that are commonly (but loosely) spoken of as the "physiological-biochemical" characteristics of the organism. Indeed, available information on the dose-response data of metabolic inhibitors with more than one site of action (fluoride, dinitrophenol, and penicillin, to cite but a few) shows that reactions essential to the polymerization involved in growth and division may be far more sensitive than reactions in the oxidative or fermentative degradations. It is undoubtedly significant that the energetic couplings and transferase reactions (the interchanges of the synthetic thruways) have been found, in the few instances thus far elucidated, to be the loci affected by the inhibitory substances.

Uncoupling of Cell Division from Cell Growth

From many observations on the induced growth of bacteria, yeasts, and algae as massive homogeneous populations of filamentous cells, rather than as normal small cells, the thesis has been devloped that growth in the absence of cell division results from the uncoupling of the division mechanism from cellular metabolism.[13-15] Efforts have been made in several directions in the attempt to identify reactions specifically involved in cellular division. In one such attempt, attention has been focused on the metabolism of filamentous mutant strains of certain yeasts. Mackinnon[22] described mutants of *Candida albicans* that were distinguished from the parent in being filamentous under conditions in which the parent grew as a budding yeast. We have studied some of these mutants and have concentrated attention on one strain (strain 806) which was found to synthesize cell mass at a rate only slightly less than that of the parent and is not distinguished from it in fermentative abilities or nutritional requirements for growth. Cultures of the mutant consist of long tubular cells that have, per unit weight, a polysaccharide composition almost identical with that of the parent. Many experiments accordingly indicated the similarity of the parent and mutant, but demonstration of the metabolic difference between the two strains that was the basis of the tremendous morphological difference between them proved difficult. A fruitful approach was found in the so-called "syntrophism technique" developed by Davis[23] to facilitate recognition of differences in loci of genetic blocks in vitamin-less bacterial mutants. The parent and mutant strains were sown side by side on an agar medium to ascertain if the parent secreted any material which might be involved in a reaction beyond the genetic block of the mutant. Cells of the mutant adjacent to the parent were, indeed, more yeastlike. Cysteine was identified as the active material secreted in traces by the parent.[10]

From many earlier studies, it was known that an important aspect of glucose metabolism for the maintenance of normal strains of *Candida albicans* in the yeast-state (*i.e.*, maintenance of cellular division and prevention of filamen-

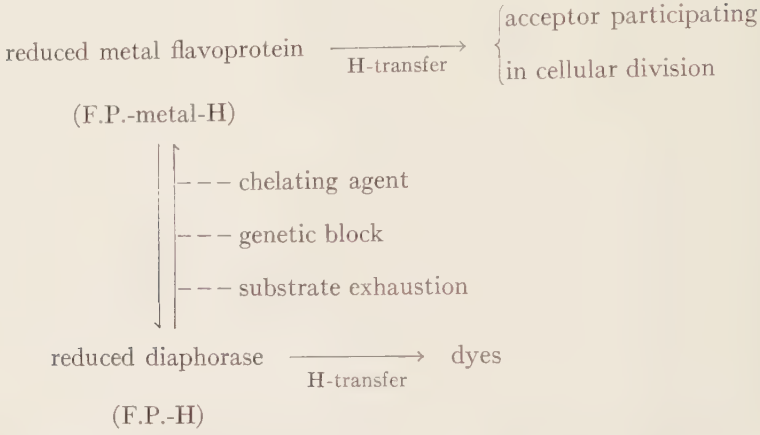
tation) lay in the metabolic generation of intracellular reducing substances that force a disulfide-sulfhydryl equilibrium (-S -S ⇌ -SH) to the right. The addition of a small amount of cysteine (CSH) or glutathione (GSH) was sufficient to prevent filamentation of the parent strain on a polysaccharide medium.[9] The existence of a cystine reductase that catalyzed the reduction of cystine to cysteine by reduced coenzyme I (generated in the course of glucose metabolism) was discovered in these yeasts.[24, 25]

These observations on the cellular division process in *C. albicans* led naturally to the conclusion that the filamentous mutant might be blocked at a reduction step. Accordingly, various oxidation-reduction indicators were incorporated into the GGY medium. On the plates containing red tetrazolium (E'_0 = −0.080 volts), the growth of the mutant was brilliantly colored (tetrazolium dyes are colored in the reduced state, in contrast to most other dyes), while that of the parent was almost colorless. Cells of the normal strain were permeated by the dye but did not reduce it; cells of the mutant reduced the dye and the insoluble, colored (formazan) dye accumulated in the cells. A biochemical difference had accordingly been found between the mutant and parent strains. Moreover, there was excellent reason to believe that some special reductive mechanism actually was associated with the cellular division process. The filamentous cells, characteristically developed in older cultures of the parent after depletion of rapidly metabolizable carbohydrate, also exhibited tetrazolium reduction.[17]

It is known that tetrazolium dyes are reduced *in vitro* by coenzyme I-linked flavoprotein catalysis of the so-called "diaphorase" type.[26] Washed, nonproliferating cells of the mutant were found to reduce tetrazolium rapidly, so that a brightly colored cell suspension was obtained after 15 minutes of incubation. Similar suspensions of cells of the parent strain slowly reduced tetrazolium so that a suspension was faintly colored after three hours of incubation. Addition of 10^{-6} M copper chloride completely inhibited dye reduction in both cell types, but the addition of 10^{-3} M concentration of a powerful metal-chelating agent (such as disodium ethylenediaminetetraacetate = Na_2EDTA) prompted a tremendous increase in dye reduction in cells of the parent strain to the extent that the parent cells became identical in this respect with those of the mutant. This finding clearly indicated that a metal must normally be associated with a flavoprotein in the parent, and that removal or tying-up of this metal by the addition of the EDTA converts the metal-flavoprotein to a metal-free, or diaphorase-type, flavoprotein. The nature of the genetic defect in the mutant is thus clearly evident as a failure of the flavoprotein in question properly to coordinate some metal, most probably iron.[17]

The evidence presented demonstrates that uncoupling of cellular division from growth (by genetic block) in the mutant or in the parent (by substrate exhaustion or by the action of certain metal-complexing substances) results from impairment to an enzymatic mechanism which is normally concerned in transport of hydrogen to an acceptor participating in cellular division. The defect results from the removal (or tying-up) of a metal from the flavin prosthetic group of a hydrogen-transporting flavoprotein enzyme, the oxidation of which is essential to the process of cellular division in yeasts. The relationships

described may be depicted in the following diagrammatic form:



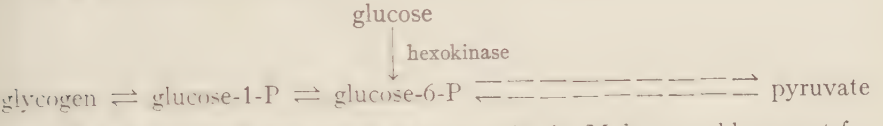
With the elucidation of the metabolic block in one filamentous yeast mutant, the groundwork is laid for an attack on other reactions participating in cellular division. Other filamentous yeast mutants are available that differ in the nature of their enzymatic "block." Identification of the genetic block in one of these mutants should reveal still another step in what is believed to be a metabolic chain responsible for cellular division. In the mutant strain described above, the block is almost certainly the initial reaction in the chain. At least, the blocked reaction is a site at which a cellular division reaction chain is coupled to the energy-yielding carbohydrate metabolism of the cell. Intensive effort is being devoted to the study of the coupling of the metal-flavoprotein to the acceptor participating in cellular division. Obviously, the nature of the "acceptor" must be learned.

Simultaneous Control of Morphogenesis and Enzymatic Activity in Microorganisms

Although it is well known to the microbiologist and to the mycologist that alterations in the environment in which an organism is grown may have a marked effect on the form of the organism, the fact that a particular morphogenetic development can be related to one component of the environment is insufficiently known. Nevertheless, the fact that small alterations in single environmental components can call forth tremendous alterations in growth form has been recorded. This literature has been reviewed[3, 4] and attention will be confined here to the reversible mycelial-yeast transformation ($M \rightleftharpoons Y$) exhibited by certain dimorphic pathogenic fungi and by many mucors.

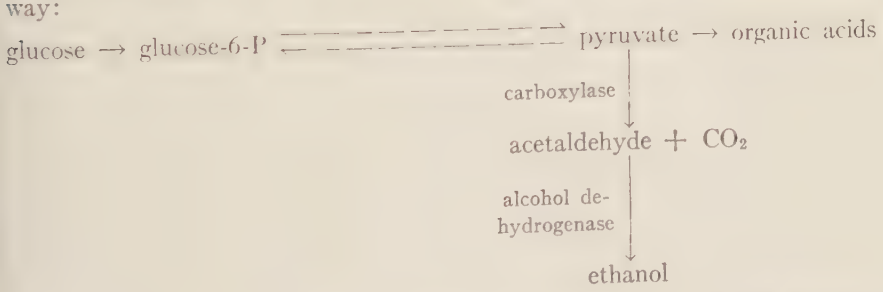
The existence of two growth types (M and Y) and the interconvertibility of these two types ($M \rightleftharpoons Y$) in an animal disease fungus was apparently first noticed by de Almeida[27] with *Paracoccidioides brazilinesis*. This organism is a budding yeast when grown at 37° C. and a filamentous fungus when grown at temperatures up to about 33° C. The fact that the $M \rightarrow Y$ transformation is

dependent only upon the temperature of incubation, and is independent of nutritional or environmental factors other than temperature, was first shown by Nickerson and Edwards.[7] Accompanying the M → Y conversion in this organism, and in *Blastomyces dermatitidis*, is the appearance of an exogenous oxidation of glucose. The M forms of both these fungi exhibit only an endogenous respiration that is not increased by the addition of glucose. Now it is unlikely that the mere difference of a few degrees in temperature of incubation can call forth the appearance of any profound difference in enzymatic constitution in the genetically identical material as exemplified by the M and Y forms of these fungi. Reverting to the concept of metabolic thruways for substrate metabolism, it is apparent that there need be only a difference in the operation of one enzyme to account for the appearance or nonappearance of an exogenous oxidation of glucose. This situation may be depicted as follows:



Failure of the enzyme hexokinase to operate in the M form could account for the lack of glucose oxidation.

In view of the morphological and physiological consequences of the failure of a metallo-flavoprotein to function in the filamentous mutant of *C. albicans*, it is apparent that the search for a single-enzyme defect as the basis for the M-state and lack of exogenous metabolism in these dimorphic fungi may well prove to be rewarding. A similar speculation might be advanced for the well-known M → Y transformation of mucors that appears to result from incubating the M form in high solute concentration. The appearance of an alcoholic fermentation simultaneously with the Y-state is quite likely not the result of the formation of the vast complex of enzymes necessary for the transformation of glucose to alcohol (improbable in the extreme), but is most likely the opening of an additional exit from the pyruvate interchange of the metabolic thruway:



The M forms of many mucors characteristically produce organic acids in quantity from glucose. The operation of a glucose thruway to pyruvate is, therefore, assured. The presence of alcohol dehydrogenase in the M forms of certain mucors has been found by Cochrane (personal communication).

One may accordingly deduce that investigation for a defect at the carboxylase-cocarboxylase level is indicated.

The inferences advanced may be in error, but they are drawn by extension of theory based on a very substantial body of facts. Perhaps they may aid in the attempt to relate morphological change to enzymatic action by indicating areas that give some promise of being loci of such relationship.

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NATURAL AND CULTURAL VARIATION IN ENTOMOGENOUS FUNGI IMPERFECTI*

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Fungi pathogenic to insects are of interest historically in view of the fact that Agostino Bassi, who originally isolated the species *Beauveria bassiana* from a diseased silkworm in 1834, is given credit (Fildes, 1951) as the first investigator to discover a "little animal" as the cause of disease.

Perhaps the most remarkable of the fungi that attack insects are species of *Septobasidium*, a genus treated monographically by Couch (1938). Its members live in a mutualistic association with colonies of scale insects, using some individuals for food and giving shelter and protection to others.

It is not certain whether the group of minute external parasites on insects, known as the Laboulbeniales, actually penetrate the skin or are purely epiphytic. Usually these minute forms are wholly superficial (Hawker, 1950) and apparently cause little inconvenience to the host.

A number of the fungal parasites of economic importance destroy the host internally. They permeate the body, block the blood stream, and finally consume all the soft parts of the insect. The skin remains more or less intact and becomes packed with closely interwoven hyphae to form a "mummy," or sclerotium.

A great deal of descriptive work has been done on the occurrence and morphology of these fungi. We are indebted to Thaxter (1888) for much of our knowledge on the Entomophthorales. Numerous papers have been published by Petch (1925, 1926, 1931, 1933). Masera (1936) and Charles (1941) have prepared comprehensive lists of insects and associated fungi.

Most of the entomogenous fungi are members of various groups within the Phycomycetes, Ascomycetes, and Fungi Imperfecti. There is also occasional reference to certain Basidiomycetes (Couch 1937, 1938; Charles 1941; Petch 1944).

Fawcett (1944) reported that entomogenous fungi are variable in their nature and in their relationship with their insect hosts. There is apparently a gradation from the strongest and most effective parasite down to those that are very weak and ineffective. Some of those classed as weak and ineffective parasites may indeed subsist entirely as scavengers. A large number of the fungi isolated from insects are believed to be scavengers only.

Members of only a few of the genera believed to be pathogenic were found in an intensive five-year survey, associated with naturally infected insect specimens‡ occurring among the numerous collections received at the Laboratory of Insect Pathology, Sault Sainte Marie, Ont. The miscellaneous insect ma-

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‡ "Naturally infected insect specimens" refers to insect material found in the field, in which the body cavity formed a sclerotium.

terial included not only collections gathered throughout Canada but also shipments received from various parts of the United States and Europe.

The isolates, with the exception of *Entomophthora* species, were asexual and belonged to the following genera: *Beauveria*, *Isaria*, *Spicaria*, *Metarrhizium*, *Cephalosporium*, *Sorosporella*, and *Hirsutella*. While seven genera (Fungi Imperfecti) were involved, the mummified larvae in most instances were *Beauveria*-infected. This fact, along with their recurrent association with insects throughout the world, suggests that *Beauveria* species may play a useful role in the control of insect pests.

A critical analysis based on numerous *Beauveria* isolates from 70 insect species and several so-called *Beauveria* species from culture collections has shown that, in general, they vary only slightly as they develop on their natural hosts. They must, in fact, be transferred to artificial media before differences that exist among them become apparent. Siemaszko (1937) apparently attached a great deal of importance to these cultural differences, for he recognized *B. globulifera** as a distinct species. He even considered that, on Polish insects, this species occurs in two forms, one giving, the other not giving, a wine-purple color to potato slabs; and that, moreover, these forms comprise a number of strains. This concept of strains was based in part on the fact that colonies of *B. globulifera* isolated from *Ips typographus* looked slightly different from those isolted from *Carpocapsa pomonella* (L.), although both imparted color to potato slabs. He ultimately concluded that the appearance of a *Beauveria* form is essentially diagnostic of insect species having the same "general adaptation." Thus color-imparting *B. globulifera* is characteristic of those insect species that live, or at least hibernate, under or in the bark of trees (bark beetles, *Carpocapsa* spp. *etc.*), whereas noncoloring *B. globulifera* is largely restricted to insect species that spend part of their life cycle in the earth, on tree roots, or on fallen trees (*Diprion*, *Hylobius*, *Acanthocinus*, *etc.*).

Siemaszko further reported that the characteristics of a given strain are retained after passage through other insect species. He found that a *B. bassiana* strain from *Coccus coccus*, after passing through *I. typographus* and *Diprion pini*, retained in culture its original diagnostic attributes.

The variations produced by isolates in the present investigation are not accorded specific status. *B. globulifera* is, therefore, regarded as a variant or strain of the type species *B. bassiana*.

Since it has been found that isolates from members of the same insect species (FIGURE 1) are just as variable as those from specimens of different species, the appearance of any specific isolate is obviously not associated with a particular group of insects. Similarly, Steinhaus (1952) reported that *Beauveria* cultures from *Pyrausta nubilalis* (Hbn.) appeared in some cases to be characteristic of *B. bassiana*, while others were atypical forms that tended to resemble *B. globulifera*. The original characteristics of various isolates undergo considerable change when transferred to different types of media, and in some cases when passed through other species of insects.

* The mycelium in *B. globulifera* develops as a loose, floccose or cottony, white to creamy mass that gives rise to a pulverulent layer of cream colored spores; in the type species, *B. bassiana*, the colonies develop a flat, mealy, finely pulverulent growth that has a chalky appearance, somewhat like the surface of a newly broken piece of chalk.

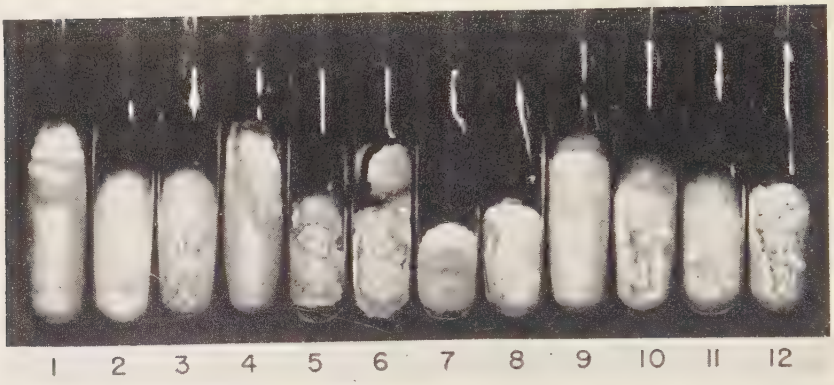

FIGURE 1. *Beauveria* cultures isolated from naturally infected *N. sertifer* on Sabouraud maltose agar showing variation in gross morphology.

Some cultures, including isolates from *Bombyx mori* L. and a *Crambus* species, grow well on Sabouraud maltose agar and poorly on blood agar base. Others, including isolates from *Venusia cambrica* Curt. and *Tolype* sp., show good to excellent spore development on blood agar base and poor spore development on Sabouraud maltose agar. Cultures, including isolates from *Sitona cylindricollis* Fahr. and *Pikonema alaskensis* (Roh.), that showed favorable spore production on Raulin-Thom medium did poorly on Czapek-Dox medium, while others, including *B. effusa* and an isolate from *Lambdina somniaria* (Hlst.), behaved in the reverse manner.

An isolate from *Neodiprion sertifer* (Geoff.), identified as *B. globulifera* on Sabouraud maltose agar, developed new characteristics resembling a typical *B. bassiana* culture when transferred to Czapek-Dox medium. It regained its original characteristics, however, when transferred back to Sabouraud maltose agar.

Single spore cultures of the *globulifera* strain from *N. sertifer*, after four generations on Sabouraud maltose agar, produced isolates that differed from the parental strain in vegetative growth, mycelial character, spore production, color on the surface, and color in the substratum. Some were flat and typically *B. bassiana;* others were extremely flocculent, typically *globulifera;* still others showed characteristics intermediate between those of the above two forms (FIGURE 2).

Isolates with characteristics identical with those of *B. bassiana* and *globulifera* and others showing characteristics intermediate between them were found on naturally infected specimens of *Choristoneura fumiferana* (Clem.), *Malacosoma disstria* Hbn., and *N. sertifer*. The numerous atypical isolates showed abnormal vegetative growth, with only poor to fair spore production.

The presence or absence of mycelial blooms and/or coremia is not a constant character. Areas of sterile, or essentially sterile, outgrowths may at times develop on otherwise typical strains of almost any *Beauveria* culture. Quite commonly, sterile outgrowths can be isolated and continued in a separate culture, where they exhibit little if any of the characteristics of the parent culture.

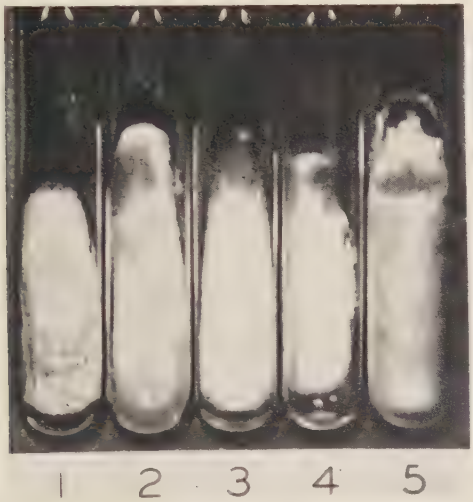

FIGURE 2. A *Beauveria* isolate (No. 1) from *N. sertifer* showing variation in gross morphology, following a series of single spore transfers (Nos. 2, 3, 4, 5) on Sabouraud maltose agar

Invariably, the mycelium at first is white but, as growth continues, the color in some of the cultures gradually changes to resemble that of the spores which develop over the surface of the cultures as they mature. The colors of the spore masses of different isolates fall fairly consistently within a narrow range of tints of the colors white, yellow with admixtures of orange, green, and red. For example, on Sabouraud maltose agar, the isolate from *Pristiphora erichsonii* (Htg.) was white, that from *Camnula pellucida* Scudd. yellow, that from *Diprion hercyniae* (Htg.) yellow admixed with orange, that from *Enypia venata* Grt. yellow admixed with green, while the isolate from *Euphydryas chalcedona* (Dbldy. & Hew) was yellow-red.

Some variation occurred in surface color when isolates were transferred to different types of media. For example, the isolate from *C. pellucida* was white on corn meal agar, light yellow on blood agar base, medium yellow on Sabouraud maltose agar, a darker yellow on potato dextrose agar, and yellowish red on Molisch medium.

Variation in surface color also occurred among *Beauveria* cultures isolated from naturally infected specimens of the same insect species. Isolates from specimens of *M. disstria*, *C. fumiferana*, and *N. sertifer* varied from white to yellow to cream to brown.

With age, the surface color of *Beauveria* cultures often becomes darker in color and assumes various shades of grayish-white, yellowish-brown, or reddish-brown.

A variation in surface color also developed when single spore cultures were established from *Beauveria* cultures. The *globulifera* strain on *N. sertifer*, originally white to pale cream, gave rise, after three generations of single spore cultures on Sabouraud maltose agar, to some isolates that were white with a red or orange tinge, and others that were a bright yellow.

A variation in the pigment production in the substratum may be brought

about by changing the nutrient composition of the medium. Much more intense coloring was developed by the *Beauveria* isolates when cultured on Sabouraud maltose agar, blood agar base, and Molisch medium, than when cultured on Czapek-Dox and Raulin-Thom media, potato dextrose, or corn meal agar. The dark bluish-green pigment that developed in the substratum when the *globulifera* strain from *N. sertifer* was grown on Sabouraud maltose agar was completely lost when the isolate was transferred to Czapek-Dox medium. The color, however, appeared again when the fungus was transferred back to Sabouraud maltose agar.

The color that developed in Sabouraud maltose agar with the isolates from *Oporinia autumnata* Gn., *Campaea perlata* Gn., and others, originally a dark purplish-red, later became a lighter yellowish-red, and finally turned a bright yellow within a period of a year. On the other hand, the isolate from *V. cambrica*, originally a bright yellow blended with red, gradually developed a darker red within a year.

The color in the substratum is not always consistent for cultures isolated from different specimens of the same insect species naturally infected with this fungus. Considerable variation occurs among isolates from *C. fumiferana*, *M. disstria*, and *N. sertifer*. The color associated with the last insect species varies from red to blue to almost black, green, red and green, and occasionally yellow. Some of the isolates from *M. disstria* that colored Sabouraud maltose agar green turned this medium red after they were passed through *P. erichsonii*.

Two variants, one of which produced a red and the other a yellow pigment in the substratum, were isolated in the course of three generations of single spore transfers totalling 60 individual spore cultures from the *globulifera* strain from *N. sertifer* (FIGURE 3). On Sabouraud maltose agar, the parent strain

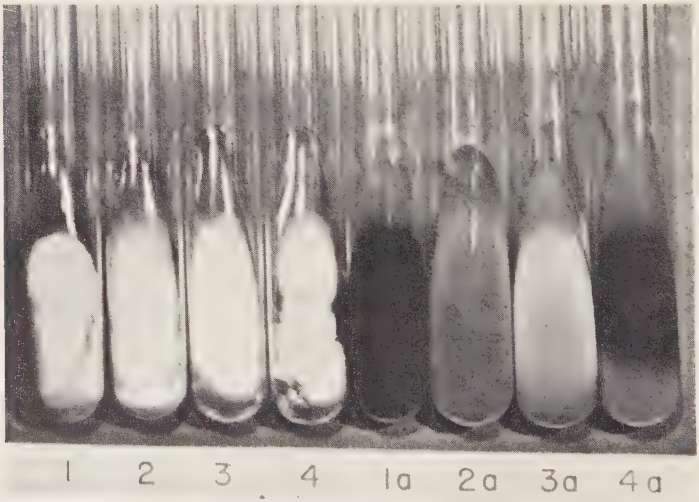

FIGURE 3. Variation in a *Beauveria* isolate from *N. sertifer* as a result of single spore transfers. Nos. 2, 3, and 4 show the variation in mycelial character, growth, and spore production of the original isolate (No. 1). The variation in color (reverse) is shown in 1a [dark green (Munsell 1915), G 3/1]; 2a (red, R 4/4); 3a (yellow, Y 8/4). Single spore isolate No. 4 in its initial growth was identical with parent culture in gross morphology and color (reverse), thereafter its cultural characteristics altered to resemble variant No. 3.

always develops a dark green pigment in the substratum. In one outstanding case, a single spore culture gave rise in successive generations to some isolates that produced a green pigment, others a red pigment, and still others a yellow pigment in the substratum. Reversion of the yellow variant back to the red strain occurred after five generations, while the red variant gave rise to some cultures identical with the original parent strain after the sixth transfer.

Variants were also obtained from a *globulifera* strain from *Archips fervidana* Clem. and a *B. bassiana* from *N. sertifer*. These two strains as well as the *globulifera* strain from *N. sertifer*, differed from each other in mycelial character, surface color, color in the substratum, and, to a lesser extent, spore production. In the course of several generations of single spore transfers, they nevertheless gave rise to variants that were identical in all respects.

Beauveria isolates do not always produce the same color in infected insects. Some strains, for example, turn *P. erichsonii* larvae red, others turn them yellow, while still other strains have no apparent effect on the color of mummified larvae. A variation in surface color among infected insects has also been noted by Vago (1951). He found that *B. mori* experimentally infected with *Scopulariopsis repens* presented all the intermediate shades between white and deep brown.

It is thus evident that the color on the surface and in the substratum is not necessarily constant on repeated subculture. The change may consist of a reduction or loss of the original color, or the acquisition of color and the power of imparting it to the medium.

Beauveria spores are borne on the threadlike apex of sporogenous cells on a series of branchlets arranged in a zigzag line. These cells, in turn, are sometimes borne directly on the conidiophores (short lateral branches) and/or mycelium, but more frequently on vesicles. The vesicles first appear as globose cells directly on the mycelium and or at the tip and along the sides of the conidiophores. The sporogenous cells and vesicles develop and multiply in mass to form compact, globose-to-oval clusters, or spore heads. The general polymorphism associated with these structural elements has been described by MacLeod (1954). It was noted that the interspecific variability was slight and of no apparent value (with one possible exception) in the differentiation of *Beauveria* isolates into species. It was found that some strains had globose spores and that others had oval spores. This difference was considered sufficient to warrant separating the strains with globose spores, as *B. bassiana*, from those with oval spores, as *B. tenella*. Even this character, however, was not invariable: *B. bassiana*, bearing mainly globose spores, had a large number of spores broadly oval, while *B. tenella*, bearing mostly oval spores, had some spores globose.

The above evidence clearly demonstrates the close relationship existing among the *Beauveria* isolates studied. Their inconstant polymorphism suggests a grouping of all the entomogenous cultures studied under one or at most two species, namely *B. bassiana** and *B. tenella*.†

Various species of *Beauveria* have been confused with *Sporotrichum*, *Botrytis*, and *Isaria* ever since the genus was named and described by Vuillemin (1912).

* *B. bassiana*—globose spores (1.0–4.0 μ), oval spores (1.5–5.5 × 1.0–3.0 μ).
† *B. tenella*—globose spores (2.5–3.0 μ), oval spores (2.0–6.0 × 1.0–4.0 μ).

These three genera can be distinguished from *Beauveria*, however, on the basis of certain morphological characteristics.

Limber (1940) named and described the genus *Tritirachium*, stating that it could be readily distinguished from other forms of Moniliaceae by the zigzag appearance of the fertile parts of the conidiophores. This particular characteristic was originally observed and described by Beauverie (1911) in relation to *B.* (=*Botrytis*) *bassiana* and *B.* (=*Botrytis*) *effusa*. Accordingly, when Vuillemin described the genus *Beauveria*, his diagnostic was essentially this characteristic spore-bearing filament.

Despite the similarity in method of spore production, there are characteristcs by which *Tritirachium* species can be readily differentiated from *Beauveria* species. In *Beauveria*, for example, the spore-bearing filament is characteristically zigzag, consisting of a series of short sharp turns or angles, whereas, in *Tritirachium* species, the turns are much less abrupt and thus appear to be more or less sinuous. This difference may be attributable to the fact that in *Beauveria* the spores are borne on short slender stalks, whereas in *Tritirachium* the spores are more or less sessile, having a flat disk-shaped basal element acting as a juncture between the broken axis of the sporogenous cell and the spore. In *Tritirachium*, moreover, the filaments are usually much longer. Some filaments have been observed that were over 110 μ in length and 0.5–1.5 μ in diameter, whereas, in *Beauveria*, none has been observed over 24 μ in length and 0.5–1.0 μ in diameter.

In *Tritirachium* the conidiophores are erect, cylindrical, hyphalike structures varying in length from 400 to 1300 μ. They occur individually, directly on the main hyphae of the colony, and become progressively thinner near the apex. The conidiophore gives rise to a whorl of three or four branches, usually at about one fourth of its normal length, a second whorl is formed 30 to 50 μ above the first, then successive whorls develop during growth, until at maturity there may be from four to ten verticils. The primary verticil may, in turn, branch into a secondary series and sometimes into a tertiary series of three or four branches. Development of the branches diminishes progressively from the base to the tip of the conidiophore, where the last, and often the next to last, whorl is composed entirely of sporogenous cells. In this way, the shrublike appearance of the colony is formed. In *Beauveria*, the conidiophores are generally in the form of undifferentiated hyphae that exist as single or branched lateral stalks (variable in size) along the axis of the main hyphae. They may be globose, oblong, or cylindrical, ranging from 2.5 to 28.0 $\mu \times 1.5$ to 2.5 μ in size. Occasionally, an extremely long sinuous mycelial type of conidiophore is formed. The conidiophores may occur individually, in twos, or in thick rosettes, but seldom in whorls along the main hyphae of the fungus colony.

There are no vesicles in *Tritirachium*, the sporogenous cells developing in whorls on the outer edge of the primary, secondary, and tertiary branches, and at the tips of the conidiophores. In some species the conidiophores appear to be simple, and the sporogenous cells in such cases develop directly on the conidiophores. In *Beauveria*, the sporogenous cells may develop directly on the mycelium and/or the conidiophores, but, as a general rule, these cells occur on globose to oblong intermediary cells or vesicles. These vesicles give rise at

maturity to sporogenous cells, secondary vesicles, or both. The secondary vesicles in turn give rise to another series of vesicles and sporogenous cells. This process is repeated several times until, in the final stages of development, there exists a more or less rounded, compact cluster of vesicles, sporogenous cells, and spores (10–80 μ in diameter, or 24 to 230 $\mu \times 20$ to 40 μ in size).

In *Tritirachium*, the sporogenous cells are variable, flask-shaped to hyphalike, ranging from 6.5 to 31.0 μ in length and 1.5 to 2.5 μ in diameter, and disposed in whorls. In *Beauveria*, the more typical sporogenous cells are globose, 1.5 to 3.5 μ in diameter. These occasionally become irregular in shape owing to crowding. The atypical sporogenous cells are quite variable in shape and size: cylindrical, flask-shaped, straight, curved, or thread-like, 7.0–28.0 $\mu \times 1.0$ 3.5 μ. A few may be extremely long, forming a sinuous mycelial type of spore-bearing filament. The sporogenous cells may occur individually, in twos, or in clusters, directly on the mycelium, on the conidiophores, or on the vesicles.

In *Tritirachium*, the spores vary from globose, 1.5 3.0 μ in diameter, to pyriform, oval, or ellipsoidal, 1.5 to 5.0 $\mu \times 1.5$ to 4.0 μ in size. In *Beauveria*, the spores vary from globose, 1.0 to 4.0 μ in diameter, to oval to ellipsoidal, 1.5 to 6.0 $\mu \times 0.5$ to 4.0 μ in size.

Cultures of some *Tritirachium* species (Beyma, 1940, 1942) develop very limited growth, which is bushlike in appearance, whereas the *Beauveria* species spread out rapidly on artificial media to form a flat or an elevated mycelial growth.

These genera also show a habitat difference in that *Beauveria* spp. are primarily parasitic on insects, whereas *Tritirachium* spp. exhibit very weak pathogenic powers, and can be regarded for the most part as saprophytic.

For the above reasons, it is concluded that *Tritirachium* species should not be transferred to the genus *Beauveria*, as suggested by Saccas (1948).

A thorough review of the literature (MacLeod, 1954) has shown that 33 alleged species have been involved in the genera *Beauveria* and *Tritirachium*. The descriptions of 14 of them are characteristic of *Beauveria*; 11 belong to the from genus *Tritirachium*; one, "*Tritirachium epigeum*", is uncertain as to genus; and seven are excluded from both. Twelve of the so-called *Beauveria* species have been reduced to strains of *B. bassiana* or *B. tenella*,* since their descriptions were not based on any outstanding characteristics. *B. stephanoderis*, *B. laxa*, *B. globulifera*, *B. effusa*, *B. sevans*, *B. doryphorae*, *B. delacroixii*, and *B. acridiorum* are regarded as strains of *B. bassiana*; while *B. densa*, *B. melolonthae*, *B. brongniartii*, and *B. shiotae* are strains of *B. tenella*.

The species *T. dependans*, *T. album*, *T. spicatum*, *T. oryzae*, *T. heimii*, *T. musae*, *T. purpureum*, *T. cinnamomeum*, *T. roseum*, and *T. hydricola* are characteristic of *Tritirachium*. Although a variability study of the so-called *Tritirachium* species has not been completed, it is evident that some are reducible to synonymy.

* *B. tenella* is regarded as a distinct species with some reservation for the following reasons: (1) it has not been isolated from any insect specimen in the present study; (2) its original description is not clearly defined (MacLeod 1952); (3) all cultures of *B. bassiana* and *B. tenella* were found to be heterogeneous in some degree. Those of the latter species were acquired from old mixed infections [illegible] it is not improbable that [illegible] tive homogeneity. (4) globose may be [illegible] *Melolontha* spp. reveal heterogeneity of this [illegible] *B. tenella* will then be treated simply as another strain of the type-strain *B. bassiana*.

B. peteloti, *B. rileyi*, *B. paranense*, *B. coccorum*, *B. coccospora*, *T. rubrum*, and *T. viannai* are characteristic of neither genus.

Although it has been shown that cultural characteristics in the genus *Beauveria* are not of sufficient importance as criteria upon which to establish species within this genus, in other entomogenous groups, such characteristics may be of value. For example, specimens of the genus *Isaria* isolated from *C. fumiferana*, *P. erichsonii*, *Arge pectoralis* (Leach), *Dioryctria reniculella* (Grt.), *Haploa lecontei* Bdv., *Pikonema alaskensis*, and a tortricid (MacLeod 1948) always displayed similar cultural characteristics on naturally infected insect material, and also when transferred to artificial media. This fungus, identified as *Isaria farinosa* (Dicks.) Fr. (Güssow 1911), invariably develops cottonlike growth with synnemata, which are bright orange in color with white tips. *Isaria farinosa* is an entomogenous fungus known to occur on Lepidoptera, Hymenoptera, Coleoptera, Diptera, Aphidae, and Arachnida.

A sterile isolate, identified as a *Spicaria* species (Siemaszko 1937), has been frequently isolated from pupating larvae of *P. erichsonii*. It produces a cottony growth tinged with orange, but does not develop synnemata. This genus is presently confused with *Isaria* and *Nomuraea*. Petch (1934), who recognized *Isaria* as a compound *Spicaria*, has suggested that the name *Isaria* be discarded in favor of *Spicaria*. According to Clements and Shear (1931) *Spicaria* is entomogenous, whereas *Nomuraea* is phytogenous in nature.

Specimens of *Neodiprion lecontei* (Fitch), *N. virginiana* Roh, *Anoplonyx* sp. probably *luteipes* (Cress.), *Lambdina fiscellaria* (Gn.), and a phalaenid, have been naturally infected with an unidentified *Cephalosporium* species. The isolates resemble each other on artificial media, and develop colonies having a powdery, white-to-pale-yellowish surface. The powdery or mealy appearance is due to innumerable conidial heads covering the hyphae. Each hypha produces at frequent intervals short lateral branches, the conidiophores. Each conidiophore bears at its apex a spherical head of conidia enveloped in mucilage. This head, when dry, appears as a glistening globule; individual conidia are not distinguishable.

Wolf and Wolf (1947) have reported that, in the American tropics, *Metarrhizium anisopliae* (Metsch.) Sorokin, the green muscardine fungus, is known to be destructive to approximately 60 species of insects. This fungus was isolated from *B. mori* at Sault Sainte Marie, Ont., and from adult wireworms (*Agriotes obscurus* L.) collected at Kentville, Nova Scotia. The isolates were similar in both instances. Rockwood (1950) refers to another species, *M. brunneum* Petch, also isolated from wireworms (*Limonius* sp.) in the Pacific Northwest.

A fungus identified as a *Sorosporella* species was obtained from several adult *Arge clavicornis* (Fab.), collected in Ontario. Its identity was based on an internal aggregation of spherical, rather thick-walled, cohering cells (resting spores) that resembled the internal spores described by Speare (1920) in his study of *S. uvella* parasitic on noctuid larvae. The infected insects were also covered with a cushion of conidiiferous hyphae, bearing spores at their tips, that seemed to have developed from small groups of secondary spores located immediately below the surface of the integument. This fungus may complete

its entire development within the body of its host, producing no growth externally. At maturity, *S. uvella* gives rise to conidiophores with bottle-shaped branchlets and an internal spore aggregate brick red in color. The *Sorosporella* sp. on *A. claviconis* gives rise to conidiophores with club-shaped branchlets and an internal aggregate of white spores. Speare (1920) has also described an unidentified *Sorosporella* species on an adult beetle, *Ligyrus gibbosus*, that produced an internal white fungal mass.

Two fungi, the one consistently isolated from larvae of *C. fumiferana* collected throughout Ontario and the other from larvae of *C. pomonella* collected in Simcoe, Ont., are believed to be species of the genus *Hirsutella*. Their identity cannot be established with certainty, since neither has been observed in a sporulating state on the natural host and they are always sterile when grown on artificial media. Synnemata, while frequently associated with infected *C. fumiferana* larvae, were found only on specimens that appeared to have overwintered in a mummified state. They do not develop readily on artificial media. Synnemata have not been observed on infected *C. pomonella* larvae, but are readily formed when the fungus is transferred to Sabouraud maltose agar. A highly viscous substance is produced by the *Hirsutella* sp. from *C. fumiferana* in yeast extract-glucose medium when incubated with shaking for 14 days at 28° C. No such substance was produced by the *Hirsutella* sp. from *C. pomonella*.

Beauveria, *Isaria*, and *Metarrhizium* species grow readily on artificial media, whereas *Hirsutella* species from *C. fumiferana* and *C. pomonella* and a *Spicaria* species from *P. erichsonii* can be established on synthetic media only with difficulty. The latter cultures are always sterile. Preliminary work concerning media of strictly defined chemical composition has shown that the *Beauveria*, *Isaria*, and *Metarrhizium* species grow readily on a simple salts-glucose medium with inorganic nitrogen as the nitrogen source (TABLE 1). Increased growth, however, can be obtained when the basal medium is supplemented with more complex materials (tryptone, *etc.*). The *Hirsutella* and *Spicaria* species require a much more complex medium with organic nitrogen as the nitrogen source.

TABLE 1

COMPARISON OF NUTRIENT REQUIREMENTS FOR GROWTH OF SOME ENTOMOGENOUS FUNGI

Fungus	Minimal medium for growth	Vitamins required
Beauveria bassiana	basal[1], inorganic[2] N	No
Metarrhizium anisopliae	basal, inorganic N	No
Isaria farinosa	basal, inorganic N	No
Hirsutella sp. (*C. pomonella*)	basal, organic[3] N, yeast extract (0.3%)	Yes[4]
Hirsutella sp. (*C. fumiferana*)	basal, organic N, yeast extract (0.3%)	Yes[5]
Spicaria sp. (*P. erichsonii*)	basal, organic N, yeast extract (0.3%)	Yes[5]

[1] Composition of basal medium: KH_2PO_4 1.5 g; Na_2HPO_4 1.5 g; $MgSO_4 \cdot 7H_2O$ 0.025 g; $CaCl_2 \cdot 2H_2O$ 0.025 g; $Fe\,NH_4(SO_4)_2 \cdot 6H_2O$ 0.001 g; $ZnSO_4 \cdot 7H_2O$ 0.001 g; $MnSO_4 \cdot H_2O$ 0.001 g, glucose 10 g, and distilled water to make 1 liter.

[2] Basal medium with 0.3 g $(NH_4)_2SO_4$ per 100 ml. medium.

[3] Basal medium with 0.3 g Na-glutamate per 100 ml. medium. Sorensen's phosphate mixture, glucose, nitrogen sources, and yeast extract were sterilized separately and added aseptically.

[4] Thiamine, pyridoxine, and other substances in yeast extract.

[5] And other factors (unidentified) that are present in the host insect.

The *Hirsutella* sp. from *C. pomonella* produced luxuriant growth on a simple salts-glucose medium containing sodium glutamate and yeast extract. On a similar medium the species from *C. fumiferana* developed a limited growth whereas the *Spicaria* species showed only slight growth.

Thiamine and pyridoxine were the only B-complex vitamins found to enhance growth of the *Hirsutella* sp. from *C. pomonella*. Other unknown substances (possibly components of nucleic acid) in yeast extract are also required for optimum growth. *Hirsutella* sp. from *C. fumiferana* and *Spicaria* sp. from *P. erichsonii* may require additional unidentified factors present in the host insect.

Host-parasite relationships show that the organisms with simple nutritional requirements (*Beauveria*, *Isaria*, *Metarrhizium*) are associated with a variety of insect species; whereas those with more exacting requirements (*Hirsutella*, *Spicaria*) appear to be limited each to a particular host species.

Summary

Asexual fungi isolated from naturally infected insect material, collected from various parts of Canada, the United States, and Europe, have been found to be members of the following genera: *Beauveria*, *Isaria*, *Spicaria*, *Hirsutella*, *Metarrhizium*, *Cephalosporium*, and *Sorosporella*.

Some variation in morphological and cultural characteristics was always associated with isolates of *Beauveria*, when established on artificial media. Isolates of the remaining genera appeared to be much more stable and, as a rule, displayed very little intraspecific variability. The question of identity has therefore proved to be a much more serious problem in *Beauveria* than in the other entomogenous genera.

A critical analysis, based on numerous *Beauveria* isolates from 70 insect species and several so-called *Beauveria* species from culture collections, has shown that the characteristics (rate of growth, amount of aerial mycelium, formation of coremia, amount of sporulation, and color on the surface as well as in the substrate) adopted by earlier investigators to differentiate species of *Beauveria* are insufficiently diagnostic. Moreover, these characteristics can be changed simply through monospore culturing or by transferring cultures from one type of medium to another. It is, therefore, concluded that only two species exist among the numerous cultures examined, namely *B. bassiana* (largely globose spores) and *B. tenella* (largely oval spores).

Of 33 alleged species attributed to the genus *Beauveria*, 14 are typical; 11 belong to *Tritirachium*; one is uncertain as to genus; and seven are excluded from both. The species of *Beauveria* lacking outstanding characteristics are reduced to strains of *B. bassiana* or *B. tenella*. The genus *Tritirachium*, which in one instance was described as synonymous with *Beauveria*, differs from it both morphologically and biologically.

Isolates from the genera *Isaria*, *Spicaria*, *Cephalosporium*, *Metarrhizium*, *Sorosporella*, and *Hirsutella* are briefly mentioned.

Beauveria, *Isaria*, and *Metarrhizium* species grow readily on a simple salts-glucose medium with inorganic nitrogen as the nitrogen source. *Hirsutella*

and *Spicaria* species require a much more complex medium including organic nitrogen.

Thiamine and pyridoxine are the only B-complex vitamins that were found to enhance growth of a *Hirsutella* sp. from *C. pomonella*. Other unknown substances in yeast extract are also required for optimal growth. A *Hirsutella* sp. from *C. fumiferana* and a *Spicaria* sp. from *P. erichsonii* are believed to require, in addition, unidentified factors present in the host.

Species of *Beauveria*, *Isaria* and *Metarrhizium*, with simple nutritional requirements, are associated with a variety of insect species; whereas species of *Hirsutella* and *Spicaria*, with more exacting requirements, appear to be limited each to a particular host species.

Acknowledgements

The author is grateful to Doctor S. A. Waksman for providing facilities to study the biology of entomogenous fungi at the Institute of Microbiology, Rutgers University, New Brunswick, N. J. He is also indebted to Doctor W. J. Nickerson for his interest and for valuable suggestions in this work.

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TYPES OF VARIATION IN ACTINOMYCETES

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Recent and current published research upon the morphology and physiology of the Actinomycetes abounds in statements and conclusions seeming to establish the great capacity of these organisms to vary. At the same time, this recognition of remarkable variability applies in at least three directions: first, in the diversity of the apparent strains which may be obtained from natural and relatively undefinable habitats; second, differences are manifested by strains or mutants which may appear spontaneously in culture or may be induced under cultural conditions through the employment of well-known agencies and procedures; third, variations occur which may be adaptive in part, often displayed by changes in the substrate or in some other environmental factor.

Variability in Fungi in General

The Actinomycetes are designedly included in the discussions today because they are generally recognized as one of the unexceptionally asexual subdivisions of the class which includes the fungi and bacteria. Being associated, more or less, with the Fungi Imperfecti, on the one hand, and with the Schizomycetes, on the other hand, the feature of variability is obviously not unusual or unexpected. With the emergence, in recent years, of their greater practical importance, the problems of speciation and variation have logically, if not necessarily, enlisted greater interest. This development has no doubt quickened the desire for exchange of ideas and observations to the end that there may be stimulated some sort of cooperation or concerted and consistent action bearing on the problems of speciation. Moreover, it has long been indicated that variability in plants increases the innate difficulties of speciation. Dobzhansky[4] has put it this way: "It is not surprising that the groups of organisms recognized as being uncommonly 'difficult' from the standpoint of delimiting species have proved to be mainly those in which asexual reproduction, apogamy, or self-fertilization are the only, or the chief, modes of propagation."

That conspicuous variability occurs in other classes of fungi, in which asexual reproduction prevails in part only, will be referred to briefly. In their authoritative work on the yeasts, Lodder and Kreger-van Rij[11] have enumerated important types of variation, as also have Henrici,[9] Skinner,[12] and Wickerham[15] in their reviews. We may note a few of these types, namely: loss of spore formation and change from smooth to rough colonies, both of which are often referred to as "degenerations"; the occurrence of spore-forming variants; loss of pigment; lethal variants; changes in biochemical properties, *etc.*

Working with *Ustilago zeae*, Stakman and associates[17] have reported that this species "definitely comprises an indefinite number of biotypes that differ either widely or slightly in every observable character or combination of characters"; and, further, after mentioning crossing and other types of varia-

* The authors are pleased to acknowledge the assistance of P. A. Nielsen, Doris Dansby, Dorothy Evans, and Marie Hauck.

bility, it is stated "Consequently, there is the most remarkable diversity within the species."

In one way or another, several mycologists have been concerned with variation and its bearing on speciation in the largely phytopathogenic genus *Fusarium*. On the basis of variation in such characteristics as color, pathogenicity, and minor structural differences, many species have been plausibly defined; but, if our interpretation is correct, the limitations of many of these species on further study have seemed illogical as well as confusing. In clearing up the confusion in certain sections of this genus, the studies of Snyder and Hansen[14, 15, 16] have been particularly significant in diminishing the number of "species" and in pointing out certain criteria and also lines of study tending to be more critically differential. Through the studies of many other investigators, for example, Hansen and Smith,[7] and Harter,[8] in the field of plant pathology and general mycology, a vast array of observations on variation in the Fungi Imperfecti is available.

In their report on aerobic mesophilic spore-forming bacteria, Smith and associates[13] under the heading "Need for classification" discuss variations and the use of characteristics in defining species with special reference to such phenomena as the following: loss of specific fermentative capacities, of pigmentation, of pathogenicity, and the like; also the capacity to exhibit different stages of growth and to vary in physiology and morphology within certain limits. It is shown that *Bacillus cereus* "possesses quite a wide range of characters and that some of its variants and biotypes have been given names as species. For instance, certain strains ferment lactose and have been called *B. albolactis*, *B. lactis*, and *B. lacticola*; others produce a yellowish-green fluorescent pigment and have been called *B. cereus-fluorescens* (not *Pseudomonas fluorescens*); others produce a rhizoid growth on agar, and if they do not ferment lactose they are *B. mycoides*, if they do ferment it they are *B. praussnitzii*." Their procedure is well indicated by the statement that " . . . before an investigator names a new species he should not only isolate and study a number of isolations but he should also determine the limits of variability of his cultures."

Further, on the side of the bacteria, it would be beyond the scope of this paper, even for the purpose of making a more complete comparison with related biological materials, to bring into this discussion the occurrence and frequency of such variations as serological types or analogous groups among human and animal pathogens. It is, however, particularly pertinent, we think, to recall, in specific relation to the genus *Bacillus* and the incompleteness of the data available in determining both taxa and the proper formulation of species diagnoses, that F. D. Chester[2] made a strong plea, as long ago as the issue of the fifth edition of Bergey's *Manual*, for additional and much needed information in reference to the characteristics of species in this genus and that, at the same time, he proceeded to show the type of work required to assure this contribution.

The Actinomycetes

Primarily, the Actinomycetes are known as soil inhabitants and, like certain other members of the "lower" fungi, including bacteria and even the yeasts, it

is usually impossible to recognize such forms and species—much less describe them—by any indirect microscopic study of the soil or other samples. That is to say, in this group especially (Actinomycetes), an organism is commonly available in nature only as fragments or as spores. Adequate study of such organisms depends upon isolation of the spore, for example, and its growth to a more elaborate vegetative stage under pure culture and other recognizedly standard conditions, both as to medium and environment. However, the investigator is completely dependent upon the organism in culture for an expression of essentially all of its properties, so that with the "Actinomycetes", the test tube, the petri dish, and the microscope are, at the outset, the most essential tools in taxonomic research, together with the requisite nutrients and certain other chemical agents.

In the recognition of strains and species, in defining strains and species, as also in studying the capacity to display variation, there is a definite limitation to the characteristics or criteria that may be employed. Fortunately, in this group of organisms, the definitive colony is characteristic and it actually behaves as though it were an individual, displaying under various conditions many distinctive growth forms and types. The simple and the complex colony types reveal the characteristics, first, of the substrate mycelium and, particularly, is it revealing with the complex colony in the form of streaks, (compare Jones.[5]) Streak cultures are even more demonstrative when cross-streaked with parallel lines to give a simple geometric figure. In such form, the colony may show more clearly the spread of growth, the color, texture, surface, and margins of the "thalloid" body resulting, the pigmentation of the substrate, and the presence of the so-called soluble or diffusible pigments which discolor or adorn the medium. Such complex colonies also disclose the developmental extent and the characteristics of any aerial mycelium and of the sporulation usually accompanying aerial hyphal development. The aerial mycelium itself may offer a great variety of patterns in respect to distribution on the colony complex, also as to color and other characteristics. In fact, the "thallose" structure is a feature of special interest and susceptible of rapid and varied response to many conditions. The characteristics of the spore-bearing hyphae and of the spores offer the morphologist many opportunities for the study of variation and should, in spite of their variability, constitute important criteria in speciation. Within the limits of this paper, it will be impossible to include even a general outline of metabolism and the specific metabolic products which may be important considerations in a further physiological and biochemical study of variations in this group.

Streptomyces lavendulae

Passing now to a consideration of variation, with the limitations indicated in the previous paragraph and as applied to a few species of *Streptomyces*, we shall begin with observations on *S. lavendulae*, a species which is apparently very common in nature and which has been long and carefully studied (see Ehrlich *et al*[6]). In the Lederle collection, there were available considerably more than 100 isolates, obtained chiefly from United States soils. For a special study, 20 of these were selected, or rather chosen more or less at random, ex

cept for two of them, designated as A2 and A3160, which were included because they are regarded by us as type material. The provisional identifications of these isolates were made on the basis of a considerable study of cultural and microscopical characteristics and by comparison with the type forms, as well as by comparison of the individual antibiotic spectra with that of a type. In general, this spectrum is that characteristic of Streptothricin, although it may to some extent be modified by the presence of one or two other antibiotics. These isolates were then tested for growth characteristics by the plate method on 11 agar substrates. Five of these substrates were chemically undefined, in that such substances as meat extract, yeast extract, N-Z-Amine and, in one case, corn steep, were employed in the media. The five just referred to are known as asparagine-dextrose-meat extract, potato dextrose, Bennett, Emerson, and Czapek-starch agars. A record of the formulation of these media is available, but it may be said that we have followed common practice. One of the media, the Czapek-Dox agar, is approximately chemically defined. Two are definitely defined, namely, the Czapek-Dox and the calcium malate agars. Three additional chemically defined media were used, but the results with these will not be considered in this paper.

In general, the growth of isolates on the Czapek-starch medium varied only from moderately good to excellent. On the other hand, growth on the Czapek-Dox medium, which utilizes $NaNO_3$ as a source of nitrogen, was not better than moderate for any one of the isolates. On the asparagine-dextrose-meat extract agar, growth varied from isolate to isolate throughout the range of moderate to excellent, but in half of the isolates, it was actually only moderate and, on two plates only, was the "excellent" level approached. The Bennett medium was apparently wonderfully well used by 13 isolates and well utilized by five others, so that only two were merely tolerant of the medium, that is, growth was moderate. In general, this result was quite unusual and more will be said of it later, in reference to the growth of other species. On the Emerson medium, there was excellent growth throughout and the variations observed were chiefly those of growth morphology. Potato dextrose agar was a comparatively good medium for all of the cultures. On the corn steep product, somewhat more than half of the isolates yielded very good growth and the others moderate. The malate medium was characteristically good but there was never much spread of the thalloid colonies or of their segments.

FIGURE 1 displays the growth behavior of six isolates including the type A3160. Two media are used, potato-dextrose agar (PDA) and asparagine-dextrose-meat extract agar (abbreviated to ADA). However, only two isolates are shown on both media. Considering colonial morphology, color, and aerial growth patterns, there are obviously accentuated variations in general and, on the bottom row, no other isolate resembles the type very closely.

On the calcium malate agar, there are illustrated growth forms of the two type cultures, A2 and A3160, along with four other isolates (FIGURE 2) all on the same medium (for a discussion of this medium, reference may be made to the work of Conn[3]). Here we note that the types are really distinctive one from another. Of the other isolates, two have solubilized the malate and two have failed to do so. Again, the aerial developments and patterns are dis-

similar and, in one case, A9460, some sectoring is displayed. If it were practicable to present the illustrations necessary, numerous comparisons of the kind described above might be made with certain isolates and each of the other media employed.

Passing now to some narrowly limited observations on hyphal characteristics of a few of the representatives of this species group, *S. lavendulae*, we may note the appearance which is commonly recorded respecting A3160 on PDA or ADA; that is, sporogenous hyphal branches which are moderately long and substantial, often with neither coiling nor conspicuous looping. However, FIGURE 9 represents a plate culture of isolate A9966, the isolate which was shown earlier in relation to colonial morphology and which was sectoring. This isolate here displays a form of looping in the hyphal tips approaching that of spiraling. It is, we think, a significant feature, particularly in view of FIGURE 10, a representation of an area on the same plate as the preceding, where apparently close spiraling is at a peak. We accordingly seem to have considerable diversity in hyphal characteristics within the one preparation. It is to be borne in mind, however, that sectoring is not infrequent in this isolate and that the variation referred to might have arisen in the culture by mutation.

That there may be considerable variation in potency (that is, in quantity) of Streptohricin production by the different strains of *S. lavendulae* seems to be generally accepted. More important, however, is the fact that several strains have been cited as producing other antibiotic products; for example, lavendulin. An analogous situation is offered in the case of *S. griseus*. This qualitative variation in the capacity of a species to biosynthesize several products coincidently, that are or may be designated antibiotics, is evidently of more frequent occurrence in the Actinomycetes than has been suspected.

Streptomyces aureofaciens

We may consider next certain growth and hyphal form variations in *S. aureofaciens*. This refers to isolates obtained from the United States as well as world-wide soil samples, such isolates being identified by ourselves and others as having certain common characteristics recognized as adhering to *S. aureofaciens*, although displaying at the same time some type of variation, and so appropriately designated "natural variants." More than 40 of these variants, out of about 100 available, were used in these tests. Likewise, other strains which represent selections from a considerable number of induced mutants have constituted an important part of the present studies. The induced mutants here referred to are all derived from a single line, that is, from A377, which happens to be the parent strain of essentially all of the strains which have been used in the commercial production of chlortetracycline (*Aureomycin*).*

On the Czapek starch medium, the *S. aureofaciens* strains which have thus far been employed in this study show the characteristic zones produced by the hydrolysis of starch around the growing organism. However, the sizes of these cleared areas are variable and not necessarily associated with the most vigorous growing organisms. Growth here is frequently of the spreading type with many strains displaying a large amount of substrface mycelium, though

* The trademark of the American Cyanamid Company for the antibiotic chlortetracycline is Aureomycin.

considerably restricted in other instances. The substrate mycelium in the different isolates varies from thin transparent to thick thallose structures variously shaded with yellow and brown tones. Aerial mycelium and sporulation are usual on this medium, yet variously styled from isolate to isolate. Abundant sectoring occurs in some strains together with a tendency to fan out from the margins of the streaks, thus giving a distinctly ragged appearance. On this medium, only one strain produces a soluble pigment, this one imparting a pinkish brown color to the agar.

On the Bennett agar, *S. aureofaciens* isolates grow well, and the linear colonies display color tones from creamy yellow through shades of olive brown or even red. The development of aerial mycelium is generally good, being distinctly heavier than that on corresponding Emerson agar, as shown later. However, certain strains show aerial development peripherally, while others display such development only on central areas of the colony, with sporulation varying from abundant to sparse. On this medium, soluble yellow to yellow brown pigments are produced by some strains. No isolate was observed to produce the thin transparent type of growth so frequently displayed in synthetic agars.

On the Emerson agar, most of the strains display a good to fairly good semi-spreading type of growth. With some of the mutant lines, however, the growth is particularly restricted in lateral expansion. The substrate mycelium reveals a wide range of colors differing markedly from strain to strain and including tones of tan and brown in addition to a strong tendency toward reddish purple hues in certain isolates. One mutant produces a substrate which, at 5 to 8 days, is a bright green (near peacock green of Ridgway), fading to olive at two weeks. The development of aerial mycelium and sporulation is much restricted on this medium and actually absent in a majority of the strains. Where such development is moderate to good the aerial growth may be scattered and whitish to gray, or it may be gray brown covering the whole surface. Diffusible pigments appear in this series in pale yellow, yellow-brown, and reddish brown tones. These pigments, however, are characteristic of only a small percentage of the strains.

In FIGURE 3, eight isolates are shown, including A377. The medium is the asparagine-glycerol agar and, on this medium, the individuality of each of the isolates is well displayed, so far as lineal colonial morphology is concerned.

Attention is directed to the yellow of isolate A1833, the unusual red brown of T3491, the spread of lobe and quadrate segments of AA804, the markings of the aerial sporogenous hyphae, especially in AA689, and the active sectoring "festival" of AB449 on this medium.

Certain mutant derivatives of A377 are displayed in FIGURE 4, and the indication is furnished that these derivatives are as diverse in characteristics as natural variants might be with habitats in South America, Japan, or the Belgian Congo. I may also refer to the orange yellow of the A377-4408 mutant as also of A377-3980, to the red of S250, the brown purple of U147, the expanded thallose colonies of A377-4413 and of A377-2655, each with its distinctive physiography, and to all of the several forms with inexpansive or narrow-band colonies, yet individualized by characteristics of hyphae and

sporulation. Many more important comparisons and parallelisms between certain characteristics of natural variants and of induced mutants will be presented in another place (Backus *et al.*[1]).

Color patterns on three media are indicated in FIGURE 5. It will be seen that each of the four isolates appears vertically on the three media. On the Emerson agar, there is a very uniform spread, high color (although quality varies), also a waxy surface with little sporing. On the Czapek-starch medium, two isolates attain only a weak and transparent, yet dissimilar growth, whereas two yield thin membranous structures with diverse surface markings. On the Bennett medium, the two at the right in the figure are alike chiefly in the absence of sporulation, while the two at the left, though differing one from the other, are both full of character.

FIGURE 6 is concerned with four mutants on the same media as represented in the preceeding figure. There is emphasized, in this case particularly, that less sporulation and high color are dominant in response to the nutritional conditions of the Emerson medium. There is the usual lighter growth on the Czapek starch. Here the Bennett agar displays variability of both general form and surface as also of the aerial characteristics of the isolates.

Variation in respect to histological features deserves detailed consideration. For the moment, however, it may suffice to refer cursorily to hyphal branches at the time of sporulation in strain A377 and to a few natural variants. In the strain mentioned, sporogenous hyphae are usually flexuous on ADA or PDA, but variation toward looping or light coiling has been noted on other media. It may be reminded that the isolate referred to is probably from 50 to 100 times removed (by "transfer") from the parent culture. Observation of six natural variants of the species, all grown on the same three media gave the following distribution of hyphal tip characteristics: coiling present, six cases; coiling absent, nine cases; no aerial mycelium, three cases. Certain relations between the parent strain, above, and some of its mutants, will be indicated in the next paper in this Monograph.[1]

It was noted in 1948 that *S. aureofaciens*, as represented by strain A377, and certain mutants of it displayed a fungistatic property, for example, against *Trichophyton mentagrophytes*.[2] Some other derivatives of A377 do not display this property; or it may be that the concentration of the agent attained on the substrates employed was insufficient to demonstrate the property. Nevertheless, it has developed that approximately 50 per cent of the natural variants tested up to the present time do possess, to a greater or lesser degree, an antifungal metabolic product. Cognizance must be taken of this and other types of biochemical variation in order that the characteristics of species may be logically defined.

Other Species of Streptomyces

Included in the genus *Streptomyces* are strains of organisms in which, typically, the sporogenous branches are borne as whorls of various complexity on rather rigid parent hyphae. Apparently the incidence of this verticillate characteristic and the constancy of other associated characters have not as yet been sufficiently followed to determine whether or not the group might logically

justify generic status. In any case, it seems to constitute a rather diverse and interesting group. According to the records available, there are perhaps more than 250 of these isolates in the Lederle Culture Collection, some unidentified, but also including such named species as *S. rubrireticuli*, *S. cinnamoneus*, *S. griseocarneus*, *S. netropsis*, and some others regarded as variants of the named forms.

For comparative variability studies, 20 of these whorled isolates were chosen. These isolates were tested on six nutrient agars following the procedure with *S. lavendulae*. We shall report briefly the results on five media, namely, PDA, Bennett, Emerson, Czapek-starch, and corn steep agars. With suitable media, most of the whorled isolates produce an uncommonly heavy growth of aerial mycelium. On PDA, Bennett, and Emerson agars, not more than two isolates failed to grow very well, to spread considerably, and to sporulate heavily. On the Czapek-starch, the group as a whole displayed less growth than those just mentioned. The growth and the sporulation on the corn steep agar were less, on the average, than on Czapek-starch.

For a comparison of isolates regarded by us as among natural variants of the *S. rubrireticuli* group of strains, FIGURE 7 displays growth patterns of two isolates on the four media indicated. While there are many characteristics in common, the nearest approach to similarity of pattern is to be found in the growth forms on the maltose, Bennett, and Emerson media. Notice, however, the narrower, bandlike growth and the reduced sporulation on the glycerol medium. On the whole, there are definable variations, that is, differences between the components of the several pairs. Such differences may perhaps serve adequately to indicate a type of variation which we find confirmed by many other comparisons in the group of 20 strains selected.

There is considerable variation in the characteristics of the whorls of branches and of the individual sporogenous units of such whorls, as in *S. netropsis* (FIGURE 11), *S. griseocarneus*, and *S. cinnamoneus* (FIGURE 12). It would seem that detailed studies of the several species and apparently numerous strains have not been made.

Material is available for a discussion of colony morphology and sporulation from the standpoint of variation in *S. griseus*, but since this species may receive liberal attention in another paper in this Monograph, we shall restrict our presentation. Instead of giving you details we may merely note that 17 strains of the species have been used on seven media and variations of considerable interest have been found, especially in the surface markings of sporulating colonies, as well as in certain color patterns.

We wish to direct your attention next to FIGURE 8, which is important in emphasizing the extent to which certain cultures may entertain variants which apparently have arisen as spontaneous mutants and may persist when transfers of spores *en masse* are made to fresh agar slants. The variant colonies are clearly seen through application of the technique of the dilution culture. Established as strain cultures, the variants display most of the usual characteristics of the group. The culture shown is a natural variant of the species *S. aurcofaciens*, isolate A2380. This culture does not commonly display recognizable sectors, nor does A4312, and certain other variants furnishing similar

evidences of variation, some with a high degree, some with lesser frequency of this variant "contamination."

Some notable cases of sectoring as variation phenomena in mycelial patterns have been referred to incidentally, but a special case of sectoring in an unidentified isolate, T3110, will be cited as giving a suggestion of an interesting "succession."

A complex streak colony of this isolate on an aging plate (14 days) with medium ADA had sporulated with the production of a stratum of gray spores. Around the edge or rim of the structure, chiefly, but not continuously, sectors were developing. These sectors no longer produced spores gray in quantity; rather, the areas were definitely blue. Such areas gave the appearance of a blue extension and partial overgrowth, as the medium became less moist. Carefully abstracting spores from a blue area and a gray area, then pouring several series of dilution or "spread" plates, it was found that the two types of colonies in their respective dishes were true to type, and there has been no reversion from blue to gray so far. Moreover, a microscopic examination of the sporogenous hyphae of the gray area displayed short, loose spirals (FIGURE 13), while a similar examination of a blue sector on the far margin of the colony afforded a picture of numerous closely coiled spirals (FIGURE 14). Antibacterial screening tests made from established cultures seemed to establish similarity of spectra, but a lower potency of the antibiotic furnished by the blue type. There may have been other variations less readily detected.

Included in the foregoing study, in order to broaden the field, were two organisms described in the literature as species of *Nocardia*. Variations in these forms were of such nature as to suggest the desirability of further comparisons before offering the observations made.

Concluding Comments

The data from an experimental study of a few morphological and ecological characteristics of a limited number of selected Actinomycetes is interpreted as confirming and further emphasizing the extent of the variability which characterizes this group of organisms. This variability is revealed in part by responses in growth and development in culture, and the evidence is strong that variations of this order exist and proceed in nature as well as in culture, essentially along parallel lines. It is perhaps obvious that characteristics which are either intrinsically morphological or physiological should be taken into consideration in the problem of speciation as applied to a group of this kind.

It has been stated frequently, in effect, and we believe it to be true, that because of inadequate study of strains, and accordingly of comprehensiveness of published specific descriptions, it has often seemed more practicable to propose a new name and description rather than face the existing hazard of "identification." Also, it would seem certain that none would wish to reduce the species concept to racial or near-biotype rank; rather, we should continue to foster a logical and a practical means of effecting order in a rather large population of variants. If all minor or single variations of morphological or developmental features, of responses to environmental changes, of differential election of nutrients, or of metabolic differences were made a basis of species

differentiation, then we might easily arrive at thousands of species where we now have tens.

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Growth responses of natural variants of *Streptomyces lavendulae* on various agars.
Figure 1. Growth of four isolates on potato-dextrose (PDA) and of four isolates on asparagine-dextrose meat extract (ADA). Two of the variants are common to both agars.
Figure 2. Growth of six isolates on calcium malate agar.
Growth responses of *S. aureofaciens* on asaparagine-dextrose agar.
Figure 3. *Natural variants*. Growth characteristics of eight isolates.
Figure 4. *Induced mutants*. Growth patterns of eight mutant strains.
Growth responses of *S. aureofaciens* on Emerson, Czapek-starch, and Bennett agars.
Figure 5. *Natural variants*. Growth patterns of four isolates on each of the three media.
Figure 6. *Induced mutants*. Growth characteristics of four strains on each of the three media.
Figure 7. Variations displayed on four media by two isolates resembling *S. rubrireticuli*.
Figure 8. Spontaneous mutation in isolate A2380, a variant of *S. aureofaciens*.
Variations in morphology of sporogenous hyphae in certain Streptomyces.
Figure 9. Hyphal tips of isolate A9966, resembling *S. lavendulae*, showing looping.
Figure 10. Hyphal tips of isolate A9966, resembling *S. lavendulae*, showing crude spirals.
Figure 11. Hyphae of isolate AA877, resembling *S. netropsis*, showing character of whorls of sporogenous branches.
Figure 12. Hyphae of *S. cinnamoneus* showing character of whorls of sporogenous branches.
Figure 13. Sporogenous coiled hyphal branches of isolate T3110, gray area.
Figure 14. Sporogenous coiled hyphal branches of isolate T3110, blue sector.

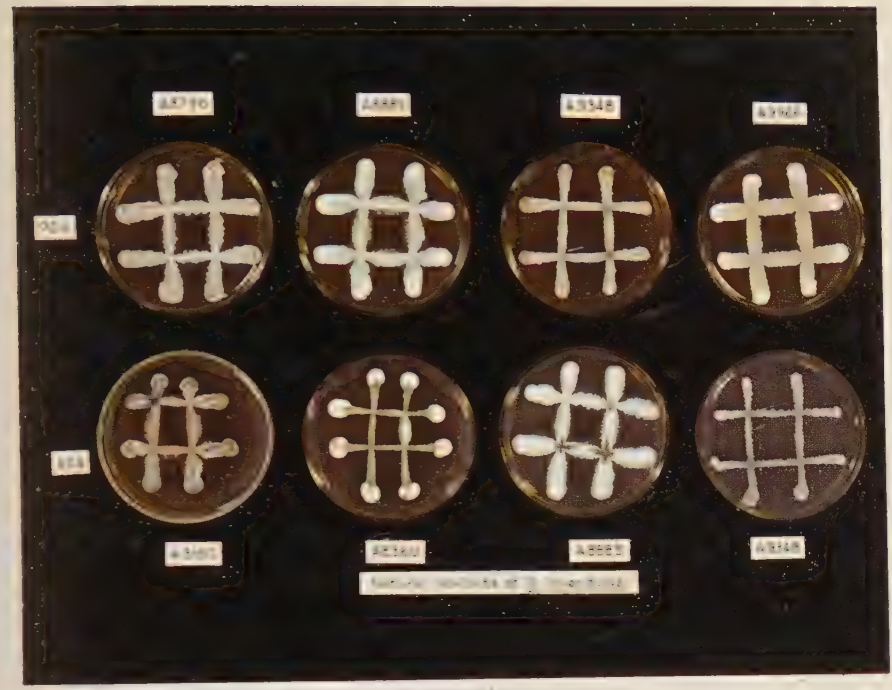
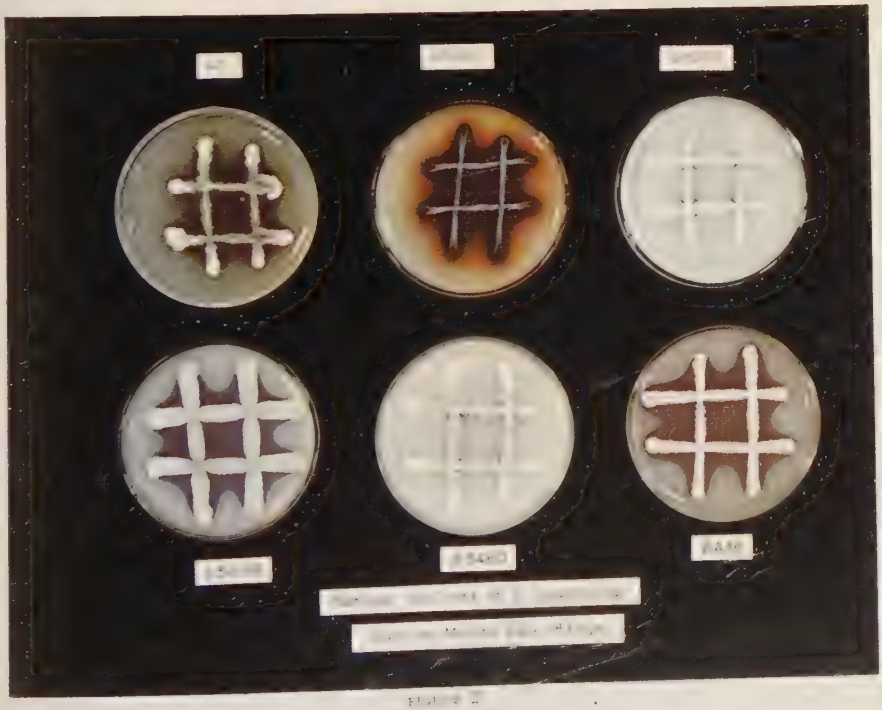


FIGURE 1



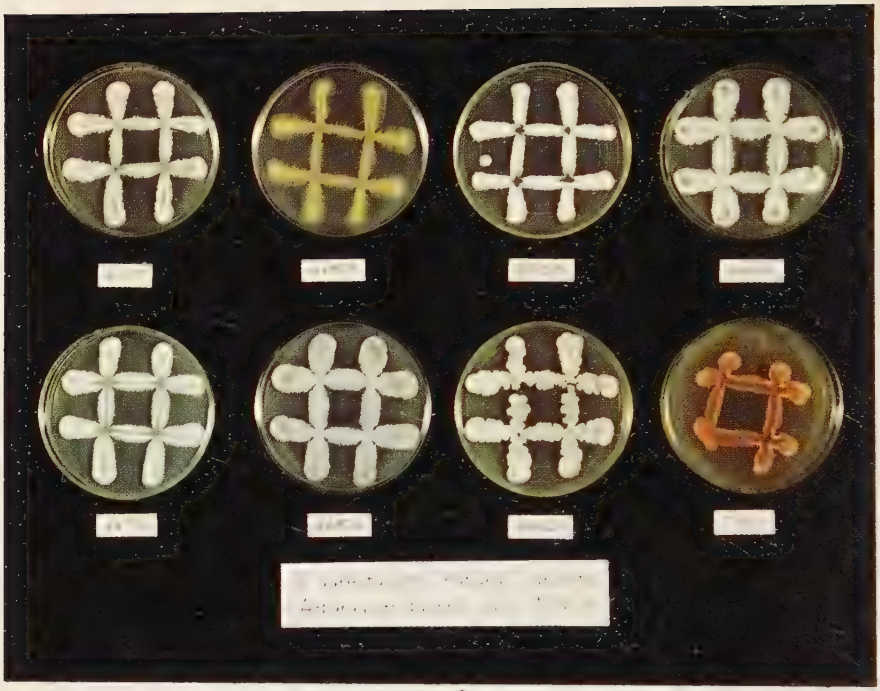


FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6

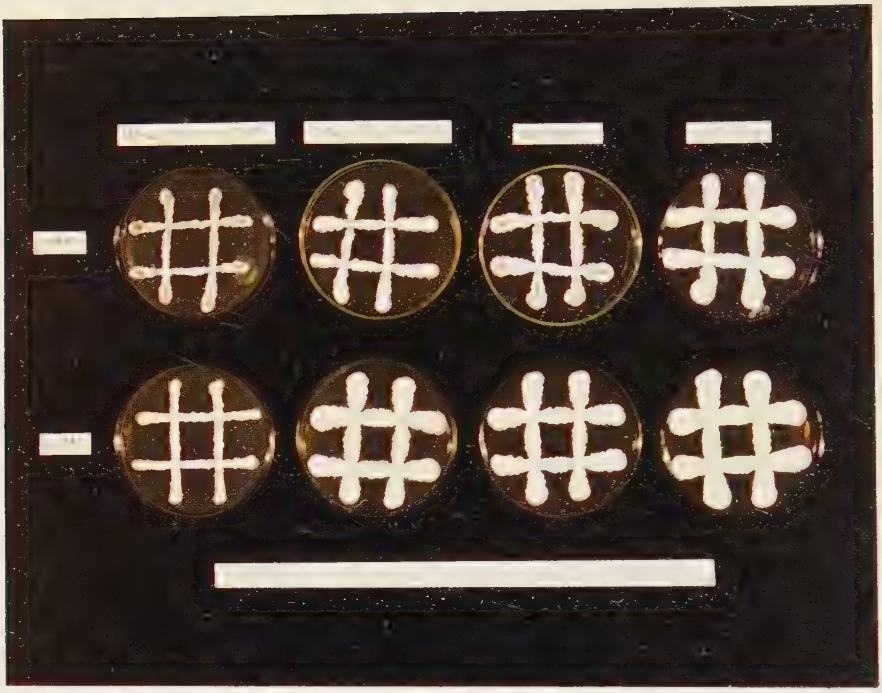


FIGURE 7

FIGURE 8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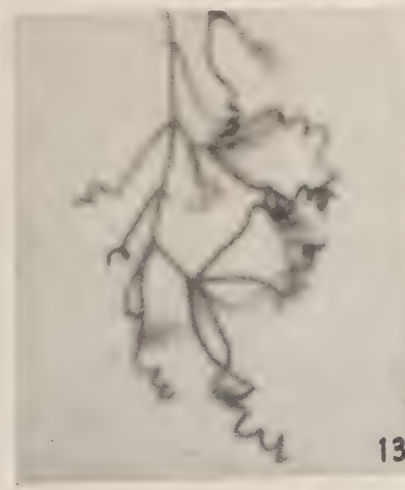
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14

VARIATION IN STREPTOMYCES AUREOFACIENS

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It is doubtful that any other genus has ever before been the subject of such intensive study as has *Streptomyces* since the discovery of streptomycin a decade ago focused attention on the potentialities which this group presented as essentially virgin territory for antibiotic "prospecting." Innumerable are the new species and new antibiotics that have been described since that time, but very few are the antibiotics which have been able to pass the severe tests of the clinician. Those that have gone on to achieve prominence as highly effective therapeutic products have carried along to fame the organisms responsible for the drugs' existence. One such combination emerging from this vast and concentrated study was the *Streptomyces aureofaciens* chlortetracycline† tandem, originally reported by one of the present authors (B.M.D.) in 1948[1] before the members of this Academy and further described by the same author[2] in United States Patent 2,482,055.

Despite the widespread attention recently given to the *Streptomyces*, the phenomenon of variation here, as in other fungi, poses persistent problems of a taxonomic nature. That others have recognized the difficulty involved in speciation in the Actinomycetes, as occasioned by the existence of extreme variation, is evidenced by the fact that, in 1945, Schatz and Waksman[6] make the comment that "Variations have caused considerable confusion in the recognition of fixed species or types for the characterization or classification of this group of organisms." In studies concerned with induced mutation and strain selection in *Streptomyces griseus* Dulaney, Ruger, and Hlavac[4] call attention to the abundance of cultural, morphological, and physiological variation which may be demonstrated in that species. During the period from the time of our first isolation of strain A377 to the present, an ever-continuing study of variation and mutation in this species has clarified the range of characteristics which *S. aureofaciens* may display.

Culture Origin

The assembled variants of *S. aureofaciens* upon which the present discussion is based have been accumulated in several ways during the last eight years. The majority of those which we designate as natural variants have been isolated from soils collected in 23 of the states in this country and in some of the countries of South America, Africa, Europe, and Asia. Certain of these soil isolates have come to us from other laboratories whose specialists have determined their identity. Other large groups of *S. aureofaciens* variants which have been used in this study are those derived from natural isolates by induced mutation. This extensive program, designed primarily to furnish mutants of superior antibiotic-yielding capacity, has seen the emergence of strains with

* The authors are pleased to acknowledge the assistance of P. A. Nielsen, Doris Dansby, Dorothy Evans, and Marie Hauck.

† The trademark of American Cyanamid Company for the antibiotic chlortetracycline is Aureomycin.

wide ranges of variation in cultural, morphological, and physiological properties as a result of treatments made with such agencies as ultraviolet radiation and nitrogen mustard.* While some few mutants have been included which were obtained from *S. aureofaciens* soil isolate A9635, a large percentage of those studied and considered here are directly descended from the original A377 line. The availability of this large group of induced mutants of common origin affords extensive opportunity for study of the occurrence of types closely paralleling certain variants isolated from natural sources.

Cultural Studies on Defined Media

Several series of agar media, both natural and synthetic, were extensively used in making comparative studies of variation in growth patterns and general macroscopic phenomena as associated with selected *S. aureofaciens* isolates. Special consideration is given here to experiments carried out with more than 50 variant strains on a series of synthetic agars. These media all had a basal composition consisting of K_2HPO_4 0.68 per cent, $MgSO_47H_2O$ 0.05 per cent, Hoagland's A-Z mineral solution 0.10 per cent, and agar 1.8 per cent. Combined with this composition were a carbon source at 1.0 per cent and a nitrogen source at 0.2 per cent. Selected for use as carbon-furnishing compounds were glycerol, maltose, and lactose. Selected as nitrogen-furnishing compounds were glutamic acid, $(NH_4)_2HPO_4$, and urea. A system of cross combining in which each of the three above-mentioned carbon sources was employed in a medium with each of the three distinctive nitrogen sources resulted in the formulation of nine separate combinations of these nutrients. The urea was sterilized by Seitz filtration and was added aseptically, where required, in proper concentration to the other ingredients after they had been autoclaved. All media were titrated to pH 6.8. Quantities of each agar were prepared sufficient to afford simultaneous study of all members of the selected group of variants, both natural and induced. The organisms were applied as spores which were streaked onto the agar plates as two parallel lines in one direction and as two parallel lines running perpendicular to the first pair. Incubation was at 28° C. in a room with comparatively constant humidity.

The gross descriptions which follow are essentially summations of observations made during the growth period, approximately three weeks, and indicate certain variations evident in the "selected group" of isolates as they are expressed under conditions of nutrient and environment as specified.

S. AUREOFACIENS *on glutamic acid agars* (FIGURES 1 and 2). On glutamic acid-glycerol agar the growth streaks are variously spreading to compact, of good depth to thin and scalelike. The substrate mycelium surface may be smooth to somewhat wrinkled or folded, and waxy to dry or crusty. Color variation in the substrate mycelium ranges from yellowish cream through various shades of yellow and brown to a distinct pink in one strain and a deep ruddy red-brown in others. Development of aerial mycelium is generally limited to the ends of edges of the streaks in most strains. However, it may occasionally be heavily developed, resulting in a complete white covering over the substrate layer at 10 to 14 days. Most strains sporulate slowly and sparingly

* Numerous induced mutants were furnished by our colleagues, Doctors J. Growich and R. Weindling.

on this medium, although a few may ultimately produce abundant spores. Where present, the spore mass may be various shades of brown through gray-brown to gray. Diffusible pigments are generally absent, but certain strains may exhibit abundant yellow to amber to red-brown shades.

On glutamic acid-maltose agar, the general habit of growth is more compact and restricted in lateral expansion than is the case where glycerol is used with this nitrogen source. Substrate mycelia show variously folded to smooth growth surfaces, while one strain displays distinct cracking at the broadened ends of the streaks. Colors of the substrate mycelia range from pale creamy tan through various shades of yellow and brown to distinct reddish tones. Development of aerial mycelium is somewhat more vigorous here than on the glutamic acid-glycerol medium. Sporulation is quite limited in most strains but may be fair to heavy, pale gray to deep gray in certain other strains. Soluble yellow to red-brown pigments are displayed by a few strains.

When cultured on glutamic acid agar, in which lactose has replaced glycerol or maltose, a large percentage of the strains exhibit extremely restricted growth with only a few exceptional isolates displaying a spreading habit. The substrate mycelium develops along the lines of the streaks as narrow bands, varying in color with the strain from creamy tan to yellow-orange to ruddy brown. Aerial hyphae and spores are developed only by a few strains and in these chiefly in a marginal position on the streaks. Diffusible pigments (yellow to ruddy brown) are evident in several strains.

S. AUREOFACIENS *on ammonium phosphate agars* (FIGURES 3 and 4). On $(NH_4)_2HPO_4$-glycerol agar, about one half of the strains studied display a spreading type of growth, the others being semirestricted in their lateral expansion. The substrate mycelium, generally in shades of yellow or brown, is rapidly obscured in most strains by the early development of abundant aerial mycelium. Reverse coloration may range, depending upon the strain, from creamy-tan through deep shades of brownish-black. Many strains produce heavy layers of spores over most of the culture surface, but especially on the expanded, fanlike tips of growth at the ends of streaks. Spore colors range from brownish tones through deep drab gray. Sectoring occurs with considerable frequency in cultures grown in this medium, and soluble yellow-brown to brown pigments are produced by a few strains.

The substitution of maltose for glycerol in the $(NH_4)_2HPO_4$ agar does not materially change the growth characteristics of most *S. aureofaciens* strains, except for the fact that there is in general a slightly greater tendency for lateral expansion of the culture along the line of the streak. Also, there is less evidence of soluble pigments being produced on the maltose medium. Reverse colors again may range from straw yellow through various tans, olives, and browns to deep mahogany. A few strains display on this medium a very narrow restricted type of growth with aerial mycelium absent or limited to peripheral areas of the streaks.

Growth on agar containing lactose and $(NH_4)_2HPO_4$ differs from that on media containing glycerol or maltose with this nitrogen source chiefly in the distinct retardation of the development of aerial mycelium and in the fact that the substrate growth is thinner and in many instances largely beneath the agar

surface. Only a few strains produce a well developed substrate "thallus." This may vary in color from cream through rich golden brown. While some strains, those which apparently cannot adequately utilize lactose, produce only a narrow, thin, colorless subsurface growth on this medium, yet a limited number give heavier growth on lactose than on agars where glycerol or maltose is used with ammonium phosphate. Several variants were observed to produce a soluble golden pigment.

S. AUREOFACIENS *on urea agars* (FIGURES 5 and 6). On urea-glycerol agar, the substrate growth is in most instances thin, spreading, frequently moist and translucent. Certain strains produce a more compact growth of considerable depth whose colors vary from creamy yellow to rosy brown. Although a few strains are able to produce aerial mycelium and brown to gray spores in abundance, most strains on this medium display a reduced development of aerial hyphae. This frequently takes the form of a series of tiny dots along the central portions of the streaks, these being white, at first, but gradually becoming cinnamon color to gray brown with age. At least three strains produce diffusible yellow to rosy brown pigments on this medium, while several strains here display a strong tendency toward sectoring.

Growth on maltose-urea agar is similar to that just described, differing chiefly in an increase in quantity of aerial mycelium and spores and in the reduction of pigment production by certain strains.

In agar containing lactose and urea, substrate growth is variously spreading to compact, appressed to raised, translucent to golden brown, smooth to wrinkled, waxy to dry. While the development of aerial mycelium is somewhat limited in many strains, certain series of mutants and some of the natural variants display strong aerial growth and heavy pale gray sporulation.

Cultural Studies on Certain of the "More Conventional" Streptomyces Media

Many of the points of variation displayed by this species on such standard agars as beef extract-asparagine-dextrose, Emerson, Bennett, and Czapek-starch or Czapek-dextrin have been discussed and illustrated by Duggar *et al.*[3] Certain other commonly used media are equally useful in demonstrating the variability which exists between strains of this organism.

Outstanding in this respect is Conn's calcium malate-glycerol agar.[7] Striking differences in appearance and behavior, from strain to strain, are evident on this medium (FIGURE 7) where the main comparatively constant features are in the development of an essentially linear, nonspreading type of substrate growth and in the limited amount of aerial development displayed, although exceptions to these conditions may be observed. Especially variable are the colors exhibited by the substrate mycelium, which may range from cream to ruddy purple with such shades as greenish-yellow, olive tan, clay color, and chestnut being not at all unusual. Sporulation, generally scanty on this medium is fairly heavy in a few strains and ranges in shades from whitish through gray-brown to gray. The most striking variable of all on this agar is the degree of clearing of the medium through solution of the calcium malate, the range of which extends from none, with many strains, to include a large

proportion of the plate with certain others. Soluble pink and yellow-brown pigments are also produced by certain strains on this medium.

Variability between strains of *S. aureofaciens* is admirably shown in a study of their development on steamed, slanted potato cylinders. Growth here is frequently thick, nodulate, cerebroid, occasionally with dry crusty or moist, glistening, unelevated surfaces. Substrate growth is variously colored with shadings generally more brilliant in the areas of greater moisture content deep in the tube. Among the many hues observable, depending upon age and strain involved, are the following: pinkish tints merging into light buff or clay color; pale to light ochraceous buff to antimony yellow; wood brown through buffy olive to avellaneous; ochraceous through tawny olive to Sanford's brown or Dresden brown; Prussian red through ocher red and vinaceous russet; bay shading into mahogany red and chestnut. All colors are gradually dulled with aging of the cylinders at incubation temperatures (colors used above after Ridgway[5]). White to tawny aerial mycelium develops in certain strains at 6 to 8 days, in others after two weeks' incubation. Many strains do not display any aerial mycelium even after 28 days. The cylinders of potato are variously darkened and colored during growth of the organism by the diffusion of brownish to yellow pigments from certain of the strains, although many of the lines do not display such pigment formation.

Results obtained with certain other standard tests commonly used in the study of *Streptomyces* organisms add to the evidence of extreme variability in this species. On Bacto-purple milk the selected natural variants and induced mutants displayed in 6 to 18 days very poor to good collars of growth in the tubes, these collars ranging in color from creamy white through various shades of yellow and brown to near red. Curd may accumulate, sometimes in distinct layers, as coagulation takes place. Clearing of the medium is variable with the strain, ranging from only semiclearing of a small layer to substantial clearing to a depth up to 30 mm. These cleared liquids show various color tints from amber through purple-red, indicating considerable variability in reaction following growth, with certain specific mutants causing the medium to become distinctly alkaline. Observations relative to the behavior of these organisms on both plain and nutrient gelatin revealed some degree of variability in growth and liquefaction from strain to strain, but, generally speaking, these tests were not regarded as being particularly useful in this specific study. Other standard tests, such as starch hydrolysis and growth on nutrient agar or nutrient broth were found to reveal only minor, subtle differentiation between strains and were, therefore, not extensively used in this project.

Natural Variant-Mutant Strain Parallelism

Of particular interest in this study of *S. aureofaciens* variants has been the history of natural isolate A4650, whose characteristics are so divergent from those of A377 as to have caused considerable debate at one time concerning the propriety of its inclusion in the species. Among the outstanding deviations which this isolate displayed was the tendency for a large proportion of the mycelium to break up into spores or elongated sporelike hyphal bodies. This situation resulted in a distinctive, characteristic behavior in shaken flask culture

because the short hyphal elements did not cling to the surface of the glass, and therefore there was no piling up of growth on the sides of the flask. Two further deviations from the situation in A377 were the marked preference shown by A4650 for glycerol as a source of carbon and the distinctive bright lemon yellow color of the substrate mycelium and reverse, a character which persists despite aging or storage at refrigerator temperatures. This isolate also possessed other characteristics which linked it to the *S. aureofaciens* group, including the capacity to produce the antibiotics chlortetracycline and tetracycline* under proper conditions of fermentation. However, it remained for the isolation of induced mutant strain A-377-4408 from an ultraviolet radiation series with the A377 line to firmly establish the relationship of A4650 to the *S. aureofaciens* group (FIGURE 8). This mutant, while differing considerably in many details from both of the natural isolates under discussion, displayed all three of the characters mentioned above as seeming to set A4650 apart and, as a result, brings into close and obvious relationship to A377 that line of the species which once seemed most remote. While the original isolate A377 was shown to have suppressive action on *Trichophyton mentagrophytes*,[1] isolate A377-4408 displays a markedly stronger antifungal activity than A377 but not greatly different than that shown by isolate A4650. Certain other mutant lines from A377, by their diversity and duplication of bizarre characteristics, have aided in the positive establishment of several other less spectacularly different natural variants as unquestioned members of the species.

Morphological Variation

While no exhaustive morphological study has been made, and the development of the spore-bearing structures has not been followed in detail, microscopic examination of growing cultures of many natural variants and induced mutants of *S. aureofaciens* has revealed a considerable degree of variability. The size of the spores or sporelike bodies varies from strain to strain, as indicated in TABLE 1, but, in general, their dimensions would fall into the range of 0.6 to 1.1 microns in diameter by 0.6 to 3.0 microns in length. However, certain of the natural variants, chiefly the previously mentioned A4650 isolate and others of similar type, and certain of the induced mutants, for example U290, display marked deviation from this range, particularly as regards length of the sporelike bodies which occur.

Although definitely influenced by medium and other environmental conditions, habits of branching, and types of sporulating hyphae observable in this species were found to be distinctly variable from strain to strain on a given medium. While under most circumstances A377 (FIGURE 9) displays primarily straight to flexuous, but occasionally hooked or coiled, sporulating hyphae, certain mutant lines derived from this isolate may show increased incidence of hooking, looping, coiling, and loose spiraling (FIGURES 10 to 12). Also, natural variant AB374 (FIGURE 13), isolated and determined at the USDA laboratory at Peoria, Ill. (NRRL B-1288), by Benedict, displays a distinct tendency to coil quite unlike A377 but not markedly different from that characterizing certain of the A377 mutant strains. Extremely evident, too, in some of the mutant

* The trademark of American Cyanamid Company for the antibiotic, tetracycline, is Achromycin.

TABLE 1

SIZES OF SPORES OR SPORELIKE BODIES IN CERTAIN STRAINS OF *Streptomyces aureofaciens*

Natural variants	Size in microns	Induced mutants	Size in microns
A377	0.6–1.1 x 0.6–1.7	A9635–32	0.6–1.1 x 1.1–2.3
A1684	0.6–1.7 x 0.6–2.9	A9635–68	0.6–1.1 x 0.9–2.9
A2021	0.6–1.1 x 1.1–3.4	NM8	0.6–1.1 x 0.6–2.3
A2380	0.7–1.1 x 0.7–2.3	S30-6	0.6–1.1 x 0.6–4.6
A4650	0.7–2.9 x 1.1–6.3	U132	0.6–1.7 x 0.7–2.3
A5156	0.6–1.7 x 0.7–1.7	U147	0.6–1.1 x 0.6–2.9
A8549	0.6–1.7 x 0.6–2.3	U288	0.6–1.1 x 0.6–2.3
AA575	0.6–1.1 x 0.7–2.3	U290	0.6–1.1 x 0.6–17.1
AA689	0.6–1.1 x 1.1–2.3	UH51	0.6–1.1 x 0.6–2.3
AB2	0.6–1.1 x 0.6–2.9	UP7	0.6–1.1 x 0.6–2.9

lines, particularly those associated with the group designated as the U290 series (FIGURE 14), is the reduction in the complexity of the tangle of aerial mycelium and the presence of a stubby, irregular type of branching not commonly observed in such an isolate as A377.

Variation in Carbon-Nitrogen Utilization

Preliminary report is made here of studies relative to the utilization of various carbon and nitrogen furnishing compounds by 12 selected natural variants and by 12 selected induced mutant strains of *S. aureofaciens* in submerged growth tests on chemically defined solutions. All media had a basal formulation consisting of KH_2PO_4 0.15 per cent, $MgSO_47H_2O$ 0.05 per cent, and Hoagland's A-Z mineral solution 0.20 per cent. In all tests of carbon furnishing compounds, the above constituents were supplemented by the addition of 0.2 per cent $(NH_4)_2HPO_4$ as the source of nitrogen and by the addition of 1.0 per cent of the specific carbon source being tested (except salts of organic acids which were used at 0.4 per cent). When tests were made with nitrogen furnishing compounds, the basal medium was amended by the addition of 1.0 per

TABLE 2

CARBON SOURCE UTILIZATION BY 12 *S. aureofaciens* NATURAL VARIANTS IN A MEDIUM CONTAINING KH_2PO_4 0.15%, $MgSO_4$ 0.05%, $(NH_4)_2HPO_4$ 0.20%, A-Z MINERAL SOLUTION 0.20%, CARBON SOURCE 1.0%

No apparent utilization by any variant	Utilization by all variants	Utilization Variable with strain		
		Positive		Negative
Sodium acetate*	Dextrose	11	Galactose	1
Sorbose	Sucrose	10	Sodium citrate*	2
Glycine	Maltose	9	Levulose	3
Mannitol	Starches	9	Mannose	3
Arabinose	Dextrin	9	Lactose	3
	Trehalose	7	MG lactate*	5
	Glycerol	6	Xylose	6
	Sodium succinate*			
	Inulin			

* Used at .4% level

cent dextrose as the source of carbon and by 0.2 per cent of the specific nitrogen-containing compound being evaluated. The reaction of all media was adjusted to an initial level of pH 6.8. Large test tubes served as the fermentation vessels, each containing 15 ml. of medium inoculated with an aqueous suspension of spores. Agitation was by a 1.25 inch stroke-reciprocating shaker in a 28° C. room. Determination of utilization was based upon growth after 96 hours incubation as measured by the volume of wet, packed cells present, following centrifugation of the contents of each fermentation tube. While this method supplies qualitative rather than quantitative evaluation, the use of adequate controls and replication attests to the validity of the results obtained.

The primary point of interest in the tabulations of carbon source utilization (TABLES 2 and 3) and nitrogen source utilization (TABLES 4 and 5) by *S. aureofaciens* variants is the comparatively large number of compounds which fall under the heading of "utilization variable with strain." The data would tend to indicate a slightly greater selectivity and variability in the group of induced mutants than among the natural variants, but the general patterns of utilization by both groups are somewhat similar. However, the degree of variation

TABLE 3

CARBON SOURCE UTILIZATION BY 12 *S. aureofaciens* MUTANT STRAINS IN A MEDIUM CONTAINING KH_2PO_4 0.15%, $MgSO_4$ 0.05%, $(NH_4)_2HPO_4$ 0.20%, A-Z MINERAL SOLUTION 0.20%, CARBON SOURCE 1.0%

No apparent utilization by any strain	Utilization by all strains	Utilization variable with strain		
		Positive		Negative
Sodium acetate*	Dextrose	11	Glycerol	1
Sorbose	Sucrose	11	Sodium succinate*	1
Glycine	Maltose	10	Sodium citrate*	2
	Starches	8	Levulose	4
	Dextrin	6	Mannose	6
	Trehalose	5	Lactose	7
	Galactose	3	Xylose	9
	Inulin	3	Mg lactate*	9
		1	Arabinose	11
		1	Mannitol	11

* Used at .4% level

TABLE 4

NITROGEN SOURCE UTILIZATION BY 12 *S. aureofaciens* NATURAL VARIANTS IN A MEDIUM CONTAINING KH_2PO_4 0.15%, $MgSO_4$ 0.05%, DEXTROSE 1.0%, A-Z MINERAL SOLUTION 0.20%, NITROGEN SOURCE 0.20%

Utilization by all variants		Utilization variable with strain		
		Positive		Negative
Glycine	L(+) glutamine	11	DL valine	1
Asparagine	Urea	11	L(+) leucine	1
DL alanine	$(NH_4)_2HPO_4$	11	DL phenylalanine	1
DL aspartic acid	$(NH_4)_2SO_4$	8	$NaNO_3$	4
D glutamic acid	NH_4NO_3	4	DL methionine	8
L(+) histidine	$Ca(NO_3)_2$			
L(+) arginine	NZ amine A			
L(−) proline	Tryptose			

TABLE 5

NITROGEN SOURCE UTILIZATION BY 12 *S. aureofaciens* MUTANT STRAINS IN A MEDIUM CONTAINING KH_2PO_4 0.15%, $MgSO_4$ 0.05%, DEXTROSE 1.0% A-Z MINERAL SOLUTION 0.20%, NITROGEN SOURCE 0.20%

No apparent utilization by any strain	Utilization by all strains	Utilization Variable with strain		
		Positive		Negative
$NaNO_2$	Glycine	11	DL Alanine	1
	Asparagine	11	L(+) Leucine	1
	DL Aspartic acid	11	L(−) Proline	1
	D Glutamic acid	9	DL Phenylalanine	3
	L(+) Histidine	8	$NaNO_3$	4
	L(+) Arginine	6	NH_4NO_3	6
	DL Valine	6	Urea	6
	$(NH_4)_2HPO_4$	5	DL Methionine	7
	N-Z-Amine A	4	$(NH_4)_2SO_4$	8
	Tryptose	2	$Ca(NO_3)_2$	10

observed from strain to strain would seem to indicate that, at present, the use as an aid to species determination of such specific physiological data as a given strain's ability or inability to utilize a particular compound (for example, lactose, xylose, or $NaNO_3$) would not seem to be applicable in the case of this organism.

Concluding Comments

It is acknowledged that we have not considered here all of the possible ways in which *S. aureofaciens* can display variability. Prominent among the variables recognized but not discussed at this time are the diverse capacities of the different strains to produce the antibiotics chlortetracycline and tetracycline under controlled fermentation conditions. Neither have we presented any hard and fast description or delimitation of the characteristics which may be associated with this species. To those familiar with the eccentricities and inconsistencies of the genus *Streptomyces*, it must be evident that any sharp and limiting description of a specific character made today may tomorrow be bridged by the emergence of some variant whose characteristics place it outside such designated limits. Rather, we have attempted to present here some indication of the extreme differences in certain characteristics which we have had occasion to encounter and observe in this species.

We recognize the difficulty attending the proper pigeon-holing into species groups of those naturally occurring strains, variants, or mutants which are particularly divergent in a substantial number of characteristics. At the same time, we consider regrettable any tendency for investigators to describe and establish new species in this already confused genus on the basis of only a few comparatively minor deviations from some well established species type, or as the result of observing the characteristics of a single specific isolate and without due consideration even of its inherent capacity to vary.

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Growth responses of *Streptomyces aureofaciens* on (A) glycerol, (B) maltose, (C) lactose agars for 22 days.

FIGURE 1. Three natural variants on glutamic acid agars.

FIGURE 2. Four induced mutants on glutamic acid agars.

FIGURE 3. Four natural variants on ammonium phosphate agars.

FIGURE 4. Four induced mutants on ammonium phosphate agars.

FIGURE 5. Four natural variants on urea agars.

FIGURE 6. Four induced mutants on urea agars.

FIGURE 7. Growth responses of eight *S. aureofaciens* induced mutants on calcium malate agar for 15 days.

FIGURE 8. Comparison of *S. aureofaciens* natural variants A377 and A4650 and induced mutant A377-4408 on (A) asparagine-dextrose-meat extract, (B) Bennett, (C) Emerson, (D) Czapek-starch, and (E) calcium malate agars for 10 days.

FIGURES 9 to 12. Variation in morphology of spore-bearing hyphae in *S. aureofaciens* A377 and in three induced mutants derived from A377 as displayed on Czapek-dextrin agar. X166. FIGURE 9. Natural variant A377. FIGURE 10. Induced mutant A377-3980. FIGURE 11. Induced mutant A377-2655. FIGURE 12. Induced mutant U290-12.

FIGURE 13. Morphology of spore-bearing hyphae in *S. aureofaciens* natural variant AB374 (NRRL B1288) on Czapek-dextrin agar. X166.

FIGURE 14. Mycelial morphology and characteristic branching in *S. aureofaciens* induced mutant U290-4 on Waksman starch agar. X166.

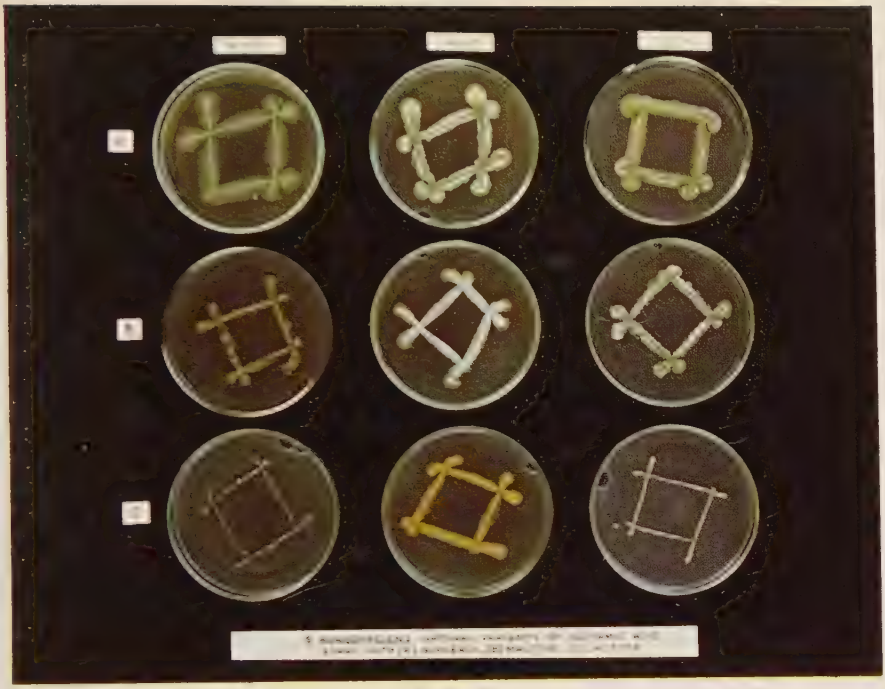


FIGURE 1

FIGURE 2

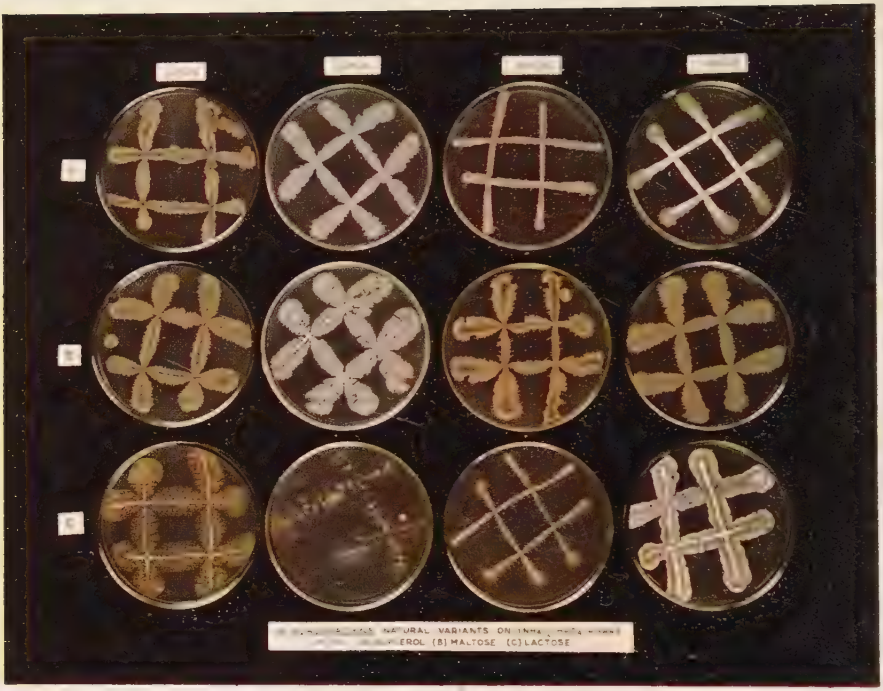


FIGURE 3

FIGURE 4

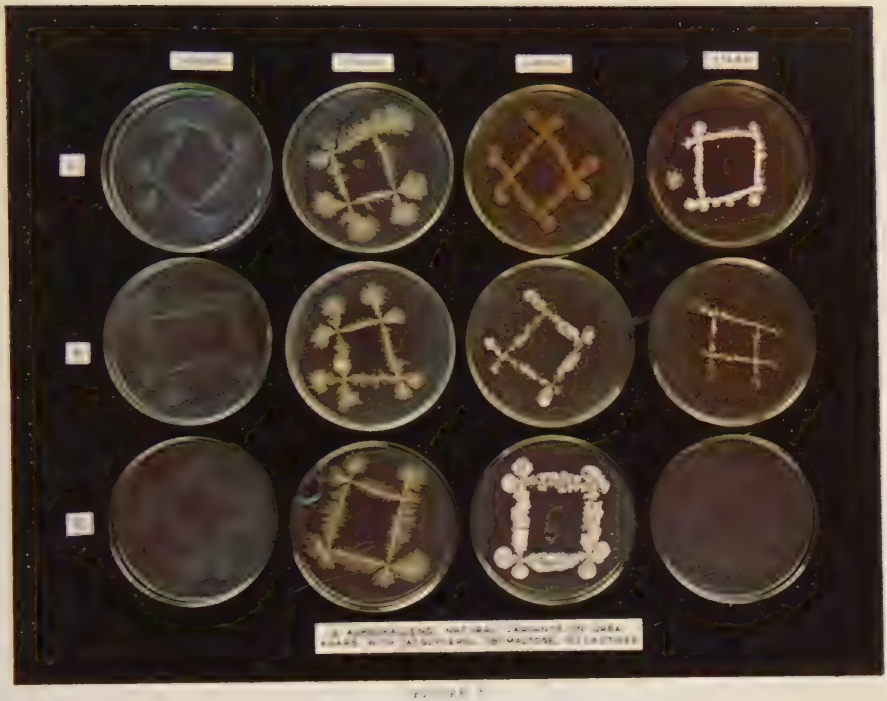


FIGURE 7

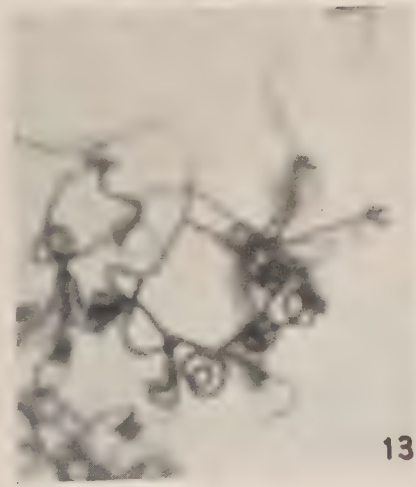
FIGURE 8


9


10


11


12


13


14

CRITERIA OF SPECIATION IN THE GENUS *STREPTOMYCES*

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It is the purpose of this paper to set forth some of the methods used in the study of *Streptomyces* and the principal criteria for speciation in this genus of the Actinomycetales. The members of the genus *Streptomyces* possess a branching mycelium which is funguslike in morphology and bacteriumlike in size, measuring generally less than one micron in diameter. Although vegetative growth is radial (actino-) and fungoid (mycete), the size of colonies usually is limited to several millimeters or less in diameter. Colonies on agar are tough, and adhere tenaciously to the substrates upon which they are growing. As colonies approach maturity, aerial hyphae arise on the vegetative mycelium, and chains of spores are produced by segmentation of the multinucleated aerial filaments. The conidiophores may branch in a simple manner or show complicated growth patterns. The spore chains, borne usually on the terminal portions of the aerial mycelium, may be long or short, simple or branched, remain straight or become flexuous, or exhibit varying degrees of curvature to form open loops, spirals, or tight coils. Different combinations of characters, including various pigments produced in the spores, in the mycelium, and sometimes in the medium, together with great diversity of colonial patterns and physiological activities make possible innumerable kinds of cultures of *Streptomyces*. The morphological expressions may be grouped into "types," which are not too numerous for a reasonable and workable classification, but when one considers further the physiological properties of substrate utilization and biochemical conversions into diverse metabolic products, the different cultural isolates become individuals, innumerable without end.

For purposes of orientation with regard to characters employed for distinguishing genera (in three families) of the order Actinomycetales, the following simple key is useful.

Key to the Genera of Actinomycetales

I. Mycelium rudimentary or absent, no spores formed ..... *Mycobacterium*
II. True mycelium produced, spores formed.
  (a) Spores formed by fragmentation of mycelium
    (1) Anaerobic or microaerophilic ..... *Actinomyces*
    (2) Obligately aerobic ..... *Nocardia*
  (b) Spores borne on aerial hyphae
    (1) Spores in chains on aerial sporophores ..... *Streptomyces*
    (2) Spores borne singly on hyphae
      a. Mesophilic, short sporophores ..... *Micromonospora*
      b. Thermophilic, longer sporophores ..... *Thermoactinomyces*

A comprehensive treatment of the Actinomycetales may be found in Bergey's *Manual*,[1] where five genera are included. The new genus, *Thermoactinomyces*,

was proposed by Waksman and Corke[2] in 1953. Much information about the morphology and physiology of Actinomycetes has been presented by Waksman[3] in an excellent book published in 1952. A guide to the classification and identification of the Actinomycetes and their antibiotics has been made available recently by Waksman and Lechavalier.[4] In these treatises, one may find many valuable references to the literature, including detailed discussions of *The Guide to the Ray Fungi*, by Krassilnikov,[5] which was published in 1944.

In the classification of Krassilnikov, as reported by Waksman and Lechavalier, 47 species are included in the key to members of the genus *Streptomyces*. The sixth edition of Bergey's *Manual* lists 73 species in the genus. According to the key of Waksman and Henrici, as presented in its latest form by Waksman and Lechavalier,[4] there are 146 species. In addition, the *Streptomyces sterilis* types are treated by these authors as being nonsporulating variants of different, named species.

Of prime importance in the keys and classification of species by Waksman and Henrici are such characters as saprophytism and parasitism, color of soluble pigments, color of the vegetative growth, aerial hyphae and spores, and micromorphology of the sporulating structures. Some consideration has been given to other biochemical properties in the descriptions of species, but little use has been made of these properties in the key. Because of the range of variation frequently observed among numerous isolates of a given species and, in view of the tendency toward mutation and selection of individual cultures maintained under continual laboratory conditions, the concept of group-species has been developed by Waksman and Lechavalier.[4] The recognition that important species and their near relatives belong in groups possessing recognizable properties, rather than in single-culture species, appears to have practical operational advantages.

Species concepts, formulated by individual workers who deal with different groups of organisms, will depend upon the experience of the investigator, whether he is a "splitter" or a "lumper," and upon the nature of the living organisms under consideration. A species of organisms which reproduce by sexual processes may be defined as a population of individuals having access to a common gene pool. There are numerous barriers and isolation mechanisms in operation here to segregate the individuals which bear specific characters. In the nonsexual (?) *Streptomyces*, a gene-pool definition of species cannot be applicable. A logical viewpoint, with certain usefulness, is that a species of *Streptomyces* should be characterized by multiple, readily recognizable, and reasonably stable properties. Of course, strict attention must be given to the history of cultures and the nature of the medium upon which they are growing; for both genes and environment are of profound importance for the phenotypic expression of diagnostic characters in the numerous isolates of *Streptomyces*, which are now under consideration in many laboratories.

Brief discussion of the principles relating to the study and classification of the species of *Streptomyces* will now be given, and certain illustrative data will be presented for the purpose of clarifying the significance of criteria employed in such studies.

(1) *Morphology of Colonies*

One of the most important characters of species in the genus *Streptomyces* is colony morphology expressed by isolates cultivated on "standardized" media. Observations of the size, shape, texture, and color of colonies growing under uncrowded conditions on chemically defined gel-media offer a fruitful approach to the classification and identification of species. Points to be noted include: (1) the appearance of the upper sporulating surface; (2) the reverse or under side, which is visible through the agar; and (3) changes in the medium brought about by the growing organism. Accurate descriptions and suitable colored photographic records are of great value for reference, identification, and comparative studies. It is suggested that striking macromorphological expressions may be observed in aging cultures growing in Petri plates for as long as two to three weeks, provided the medium is not allowed to become too dry for continued growth and development. Old cultures of many species show characteristic differences in size, zonation, spreading, and sectoring of colonies. Kodachrome or Ektachrome slide collections and dried herbarium specimens of authentic cultures, grown on defined media, can be preserved for future studies. We have been able to make numerous Kodachrome transparencies of colonies representing many species, but because of the cost of reproducing colored illustrations, none of these is shown here.

(2) *Pigmentation*

The pigments produced characteristically by numerous isolates of *Streptomyces* grown on favorable media are seen in their vegetative growth, in the spore masses, and in the medium around colonies. Numerous factors, *e.g.*, the composition of medium, amount of certain constituents present, pH during the growth period, and genotype, influence the kind and amount of pigment formed. The extent to which the brown melanin may be formed in agar cultures accordingly depends, to a considerable degree, upon the composition of the medium and on the concentration of tyrosine and of certain other nitrogenous substances, as well as on the genetic constitution of the organism. It can be readily demonstrated that production of brown pigment varies directly with increasing concentration of casein hydrolysate added to a basal mineral medium containing soluble starch. Although tyrosine appears to enhance the production of melanin,[6] it should not be overlooked that numerous isolates from soils are able to produce their own tyrosine and melanin when grown upon media containing nitrogen in inorganic form available for growth.

The influence of changing pH upon pigmentation during the course of growth needs to be emphasized, as do the wide variations in color expressed by a given isolate when cultivated upon synthetic media containing different sources of nitrogen and carbon. Since a great deal of importance has been attached to pigment formation as a criterion for speciation by Krassilnikov,[5] Waksman and Henrici,[1] Hesseltine,[7] Lindenbein,[11] and others, it is essential to be certain of the nature of the substrate and conditions of growth when making observations on such easily altered characters as spore color, reverse pigmentation, or diffusible pigment in the agar. When due consideration is given to cultural

conditions, it appears that one of the most valuable criteria for setting up major groups in the genus under consideration is color of the aerial hyphae and spore masses. Some of the outstanding "spore" colors of species groups are white, grey, pink, brown, blue-green, yellow, and black.

(3) *Microscopic Morphology*

One of the most stable expressions of a species relates to its reproductive morphology. Among the microbes, various gene mutations, transformations, and adaptations leading to alterations in biochemical properties may be readily observed. In contrast, genetic changes which lead to marked alterations in the basic microscopic morphology are less frequently observed. Induced and spontaneous biochemical mutants are commonplace. New species with altered form and function seem not to be easily invented by mutational tricks performed in the laboratory. It is suggested, therefore, that microscopic, morphological characters should receive important consideration in studies of speciation.

Cultures suitable for microscopic examination may be prepared simply in microslides for study under high magnification, or the growth formed on agar Petri plates may be examined satisfactorily in surveys under a low power microscope. Special media, with reduced carbon and nitrogen, may be employed in an effort to limit the amount of vegetative growth, so as to reveal readily the character of the aerial sporophores.

When one examines thousands of isolates of *Streptomyces* cultivated on agar media, the patterns of reproductive structure suggest a comparatively small number of morphological species groups. The aerial mycelium may take the form of short or very long hyphae, being either simple or extensively branched. The branching habit may be monopodial, sympodial, verticillate, or irregular. Sporophores vary over a wide range, from simple, short hyphae, occurring singly or in clusters, to branching cluster and compound whorls. Individual sporophores may be straight or curved or wavy, or may show a tendency toward forming loops and spirals. Coiling may take place over a considerable portion of the sporophore and spore chain, or the spiral may be limited to the apical part of the filament. In some cases, the spirals are very tightly coiled. Several illustrations of the reproductive morphology are shown in FIGURE 1. It is readily appreciated that the photographic recording of microscopic morphology is fraught with technical difficulties. Drawings permit interpretation of three-dimensional figures on a plane surface, but this kind of exposition is subjective, and dependent upon the intelligence and skill of the scientist-artist. A few examples of micromorphology, represented by several species-groups, are shown in TABLE 1. We are now engaged in a comprehensive survey of numerous isolates with a view toward the greater use of micromorphological characters in classification. The recognition of such characters in Bergey's *Manual*, and the incorporation of these characters into the key to species by Waksman and Lechevalier[4] seem to be logical

Although reproductive morphology is an important and relatively stable character for diagnostic work, still it is possible to demonstrate variability of sporophore and spore chain morphology in relation to varied substrates and

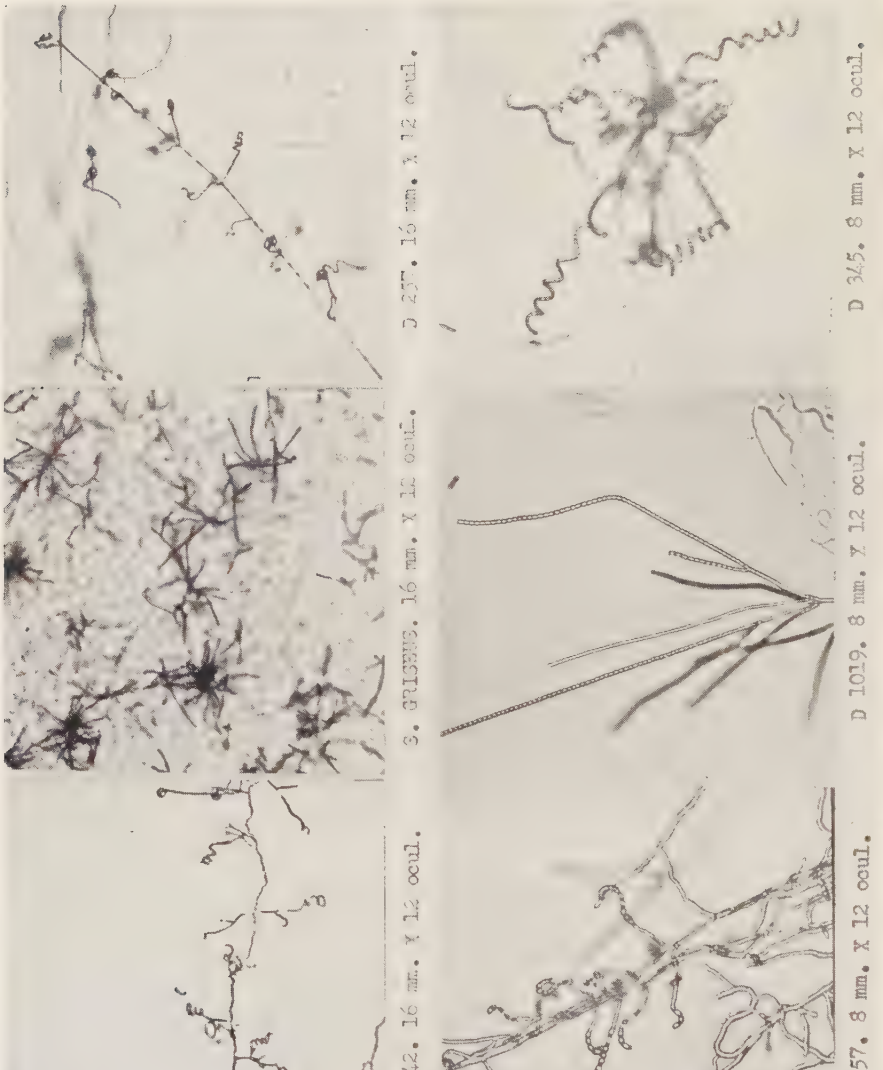

FIGURE 1. Photomicrographs illustrating some types of micromorphology in *Streptomyces*. Magnification of upper row × 310, lower row × 620.

TABLE 1

SOME ILLUSTRATIVE TYPES OF MICROMORPHOLOGY REPRESENTED IN CERTAIN SPECIES GROUPS OF STREPTOMYCES

Species group	Micromorphology of sporophores
S. albus	Long, spore chains spiral.
S. antibioticus	Straight, arranged in clusters or broomlike.
S. flavus	Long, spiral-shaped.
S. griseus	Straight or flexuous, often in tufts.
S. lavendulae	Branches not flexuous, often forming loops and loose spirals.
S. reticuli	Verticillate, spore chains spiral or straight.

conditions for growth. An illustration of some effects of varying the carbon source for three species of *Streptomyces* grown in agar plates containing the basal medium of Pridham and Gottlieb[8] is shown in FIGURE 2. Typical groups of sporophores were formed by *S. aureofaciens* growing on media containing 1 per cent of maltose, galactose, or sucrose, but atypical development occurred in media where inulin, sorbitol, or rhamnose were present. *S. griseus* developed typical clusters of sporophores in media with arabinose, raffinose, or inositol, but there was a stunted appearance on substrates of sorbitol, citrate, or succinate. *S. rimosus* produced well-developed spiral spore chains on levulose, dextrose, or sorbitol, but failed to develop in a normal way when given inulin, rhamnose, or citrate. In general, it may be said that if a medium provides nutrients adequate for good growth, the developmental micromorphology is usually typical of the genetic type.

(4) *Carbon Nutrition*

Growth and development of *Streptomyces* cultures on different carbon compounds in synthetic media have been studied by many investigators. A useful basal medium is that proposed by Pridham and Gottlieb,[8] who explored the value of carbon nutrition in taxonomic studies. The composition of this medium is as follows:

$(NH_4)_2SO_4$	2.64 gm.
KH_2PO_4	2.38 gm.
K_2HPO_4	5.65 gm.
$MgSO_4 \cdot 7H_2O$	1.00 gm.
$CuSO_4 \cdot 5H_2O$	0.0064 gm.
$FeSO_4 \cdot 7H_2O$	0.0011 gm.
$MnCl_2 \cdot 4H_2O$	0.0079 gm.
$ZnSO_4 \cdot 7H_2O$	0.0015 gm.
Difco agar	15.00 gm.
Distilled water	1000 ml.

The desired compounds, which are to be tested as sources of carbon, may be incorporated at the 1 per cent level, or on the basis of molar equivalence or equal carbon content. Sterilization procedures are important with reference to maintenance of chemical stability; hence, filtration or separate heating in aqueous solutions is required for some compounds. The isolates may be grown in culture tubes or in Petri plates. If the experimental procedures are well

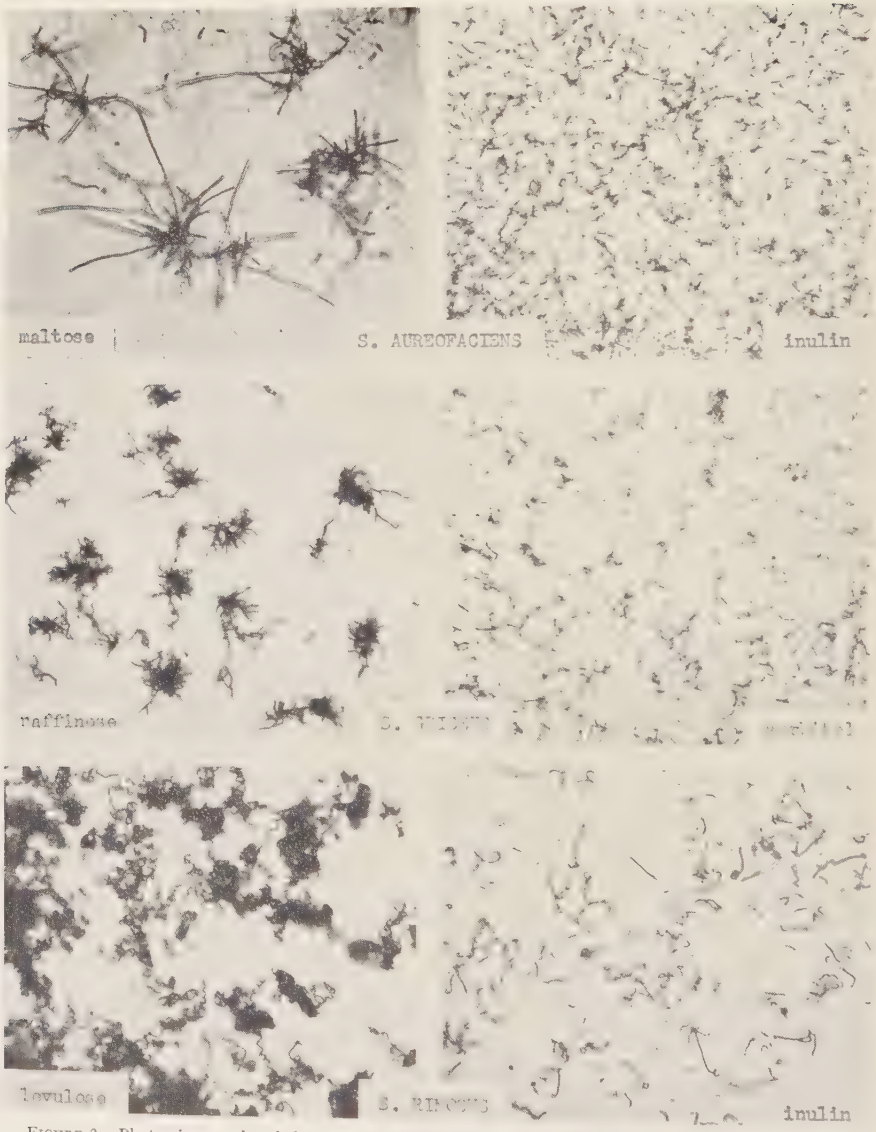

FIGURE 2. Photomicrographs of three species of *Streptomyces* illustrating variation of micromorphology in relation to different kinds of carbon compounds supplied in mineral salts agar medium. Magnification × 310.

planned in agar plates, it is possible to determine not only growth responses, but also macromorphology and micromorphology, pigment production, hydrolysis of substrates, antibiotic production, and other properties.

For the purpose of determining relative availability of different carbon sources for growth, 19 compounds were tested with 100 isolates grown in agar slants and with 30 isolates grown in Petri plates. The rare carbohydrates were obtained from Pfanstiehl, and other compounds of high grade were pur-

TABLE 2

UTILIZATION OF DIFFERENT SOURCES OF CARBON BY ISOLATES OF STREPTOMYCES GROWN ON AGAR SLANTS (100 CULTURES) AND IN PETRI PLATES (30 CULTURES). DATA EXPRESSED AS PER CENT OF ISOLATES WHICH SHOW GOOD, POOR, OR NO UTILIZATION OF INDICATED COMPOUNDS

Name of Compounds	None (0)		Poor (± to +)		Good (++ to ++++)	
	Slants	Plates	Slants	Plates	Slants	Plates
Cellobiose	2	0	11	0	87	100
Galactose	0	0	15	7	85	93
Maltose	5	0	14	7	81	93
Mannitol	16	23	18	0	66	77
Levulose	4	0	31	23	65	77
Arabinose	4	7	34	10	62	83
Rhamnose	27	43	26	20	47	37
Na-succinate	4	7	52	7	44	66
Xylose	8	7	56	23	36	70
Lactose	17	17	47	17	36	66
Inositol	32	67	33	10	35	23
Salicin	20	20	54	43	26	37
Sucrose	38	53	39	20	23	27
Na-citrate	18	20	61	27	21	53
Raffinose	47	70	38	17	15	13
Inulin	36	33	53	47	11	20
Sorbitol	47	77	52	17	1	6
Dulcitol	59	90	41	10	0	0
Cellulose	76	—	24	—	0	—

chased from reputable dealers. After incubating at 30° C. for 10 days, records of growth were recorded as none, poor, or good, over and above the growth made on a control, basal agar medium, to which no special carbon source was supplied. The data of this experiment, expressed in per cent of responses, are shown in TABLE 2. Cellobiose, galactose, and maltose are excellent sources of carbon for the majority of isolates, while sorbitol, dulcitol, and cellulose are poor. Carbon sources which provide data for clear separation of isolates into groups are of general diagnostic value. Other compounds contributing to growth of only a few isolates, or still others which are available to all but a small number of cultures may give special aid in identifying certain strains.

In another survey, 1799 isolates were tested for positive growth on six compounds supplied singly as the source of carbon in Pridham and Gottlieb basal medium. Agar discs of each medium were employed as substrata in Petri plates, and growth was estimated by visual observation. The scores of this survey are summarized, according to four patterns of response (00, 0+, +0, and ++) to inositol and rhamnose, in TABLES 3 to 6.

A summary of the six most numerous patterns of carbon utilization, occurring in 73 per cent of all isolates, reported in TABLES 3 to 6, is presented in TABLE 7. As a result of such preliminary surveys, it appears that differential utilization of carbon compounds may provide data of some diagnostic value.

Additional tests were made with named cultures obtained from collections of *Streptomyces* in the United States, including the American Type Culture Collection, Northern Regional Research Laboratory, and certain industrial laboratories. Relative utilization of 14 different carbon compounds by 10

TABLE 3

UTILIZATION OF DIFFERENT SOURCES OF CARBON BY 630 INOSITOL-NEGATIVE AND RHAMNOSE-NEGATIVE ISOLATES OF STREPTOMYCES

Ino.	Rha.	Man.	Inu.	Raf.	Sorb.	Number
0	0	0	0	0	0	343
0	0	+	0	0	0	228
0	0	+	0	+	0	20
0	0	0	0	+	0	17
0	0	0	+	+	0	14
0	0	0	+	0	0	6
0	0	+	0	+	+	2
						630

(Symbol 0 signifies growth poor or none; + means good growth)

TABLE 4

UTILIZATION OF DIFFERENT SOURCES OF CARBON BY 334 INOSITOL-NEGATIVE AND RHAMNOSE-POSITIVE ISOLATES OF STREPTOMYCES

Ino.	Rha.	Man.	Inu.	Raf.	Sorb.	Number
0	+	+	0	0	0	176
0	+	0	0	0	0	103
0	+	0	+	+	0	15
0	+	+	+	0	0	15
0	+	+	0	+	0	14
0	+	0	+	0	0	5
0	+	+	+	+	0	4
0	+	0	0	+	0	2
						334

(Symbol 0 signifies growth poor or none; + means good growth)

TABLE 5

UTILIZATION OF DIFFERENT SOURCES OF CARBON BY 261 INOSITOL-POSITIVE AND RHAMNOSE-NEGATIVE ISOLATES OF STREPTOMYCES

Ino.	Rha.	Man.	Inu.	Raf.	Sorb.	Number
+	0	0	0	0	0	74
+	0	+	0	+	0	63
+	0	+	0	0	0	49
+	0	+	0	+	+	46
+	0	0	0	+	0	20
+	0	+	0	0	+	7
+	0	+	+	+	0	2
						261

(Symbol 0 signifies growth poor or none; + means good growth)

named species of *Streptomyces*, chosen at random from a long list, is shown in TABLE 8. The patterns of utilization vary, with the different compounds, from general availability of cellobiose to extremely limited use of sorbitol by these cultures.

TABLE 6

UTILIZATION OF DIFFERENT SOURCES OF CARBON BY 574 INOSITOL-POSITIVE AND RHAMNOSE-POSITIVE ISOLATES OF STREPTOMYCES

Ino.	Rha.	Man.	Inu.	Raf.	Sorb.	Number
+	+	+	0	0	0	275
+	+	+	+	+	0	184
+	+	+	0	+	0	54
+	+	0	+	+	0	25
+	+	0	0	0	0	10
+	+	0	+	0	0	9
+	+	+	+	+	+	9
+	+	+	0	+	+	4
+	+	+	0	0	+	4
						574

(Symbol 0 signifies growth poor or none; + means good growth)

TABLE 7

THE SIX MOST NUMEROUS PATTERNS OF CARBON UTILIZATION OCCURRING IN 73 PER CENT OF ABOUT 1,800 ISOLATES

Ino.	Rha.	Man.	Inu.	Raf.	Sorb.	No.	Pct.
0	0	0	0	0	0	343	19
+	+	+	0	0	0	275	15
0	+	+	0	0	0	228	13
+	+	+	+	+	0	184	10
0	+	+	0	0	0	176	10
0	+	0	0	0	0	103	06
						1309	73

(Symbol 0 signifies growth poor or none; + means good growth)

It should be pointed out that representative isolates of some species show marked differential responses to special carbon compounds. An example of the high degree of selection by inositol is shown in FIGURE 3. In conjunction with such experiments on the nutritional relations of species, hydrolytic actions on soluble starch, Ca-malate, and other special carbon substrates can provide additional valuable information at the time of taking growth records. Also, profound variations in pigment production, associated with the development of species on different substrates, may be profitably observed in the same cultures. Just one specific example will suffice to illustrate the variation of color in relation to substrates. A fresh soil isolate, designated as D 59, was observed to produce a deep lavender-colored spore mass when grown on soluble starch agar. However, when cultivated on Ca-malate agar, the colonies were yellow, and on salicin they were white. Very often, too, the metabolic products produced in different media may alter the pH in such a way as to affect the color of certain pH-sensitive, indicator pigments of *Streptomyces*.

(5) *Nitrogen Nutrition*

Utilization of nitrogenous compounds by numerous isolates of *Streptomyces* has been studied with similar methods and for the same general reasons. In

TABLE 8

UTILIZATION OF DIFFERENT SOURCES OF CARBON AND NITROGEN BY SELECTED STRAINS OF TEN SPECIES OF STREPTOMYCES

Carbon Sources	*S. venezuelae* (P.D. A65c)	*S. fradiae* (NRRL B-1195)	*S. lavendulae* (NRRL B-1259)	*S. aureofaciens* (NRRL B 1287)	*S. antibioticus* (NRRL B-546)	*S. rimosus* NRRL 2234)	*S. griseus* NRRL B 1070)	*S. albus* (ATCC 3004)	*S. scabies* (ATCC 43)	*S. griseolus* (ATCC 3325)
Xylose	++++	++	0	++	+	+	++	+	+++	+++
Rhamnose	++++	0	0	0	+++	0	+	+	+++	0
Maltose	++++	++	+++	++	++	++++	++	+	+++	+++
Lactose	++	++	0	0	++	++++	+	+	+++	+++
Cellobiose	++++	++	+++	++	++	++++	++	++	+++	+++
Sucrose	0	0	+	+++	0	0	0	+	0	0
Raffinose	0	0	0	0	0	++	0	+	0	0
Inulin	0	0	0	0	0	0	+	+	++	0
Sorbitol	0	0	0	0	0	++++	0	+	+	0
Mannitol	0	0	0	0	++	++++	++	++	+++	0
Inositol	0	0	0	0	+++	++++	0	+	0	0
Salicin	+++	+	0	0	0	0	+	+	+	++
Na-citrate	+	0	++	0	+	0	++	++	++	+++
Na-succinate	+	+++	++	0	+	+	++	+	+++	+++
$NaNO_2$	0	0	0	0	0	0	0	−	−	0
$NaNO_3$	+++	+++	+++	0	+	+	++	+	+	0
Leucine	++	+	++	++	0	++	++	+	+	+
Isoleucine	++	++	++	++	+	0	+	+	0	++
Tyrosine	0	0	0	0	0	0	+	+	++	0
Tryptophan	0	+	0	0	0	+	0	+	++	0
Hydroxyproline	0	+	++	++	0	0	0	++	0	0
Methionine	+	++	+	0	0	+	++	+	0	++
Glutamic Acid	+++	+++	+++	+++	++	+	++	++	++	+++
Guanine	+++	+++	++	0	++++	++	+++	−	−	+++
Nucleic Acid	+++	++	++	+	+++	++	++++	−	−	+++

(Symbol 0 signifies no growth; — means not tested; increasing use represented by + to ++++.)

PLATE 1. Growth responses of 12 species of *Streptomyces* inoculated on inositol mineral salts agar medium. The responses to individual carbon sources sometimes vary among isolates of a species

TABLE 9

UTILIZATION OF DIFFERENT SOURCES OF NITROGEN BY ISOLATES OF *Streptomyces* GROWN ON AGAR SLANTS (100 CULTURES) AND IN PETRI PLATES (30 CULTURES). DATA EXPRESSED AS PER CENT OF ISOLATES WHICH SHOW GOOD, POOR, OR NO UTILIZATION OF INDICATED COMPOUNDS

Name of compounds	No growth (0)		Poor growth ± to +		Good growth ++ to +++)	
	Slants	Plates	Slants	Plates	Slants	Plates
Glutamic Acid	0	0	4	17	96	83
Glycine	4	0	15	35	81	65
Aspartic Acid	0	0	20	35	80	65
NH_4Cl	4	0	17	28	79	72
Proline	9	0	17	28	74	72
Alanine	6	0	22	17	72	83
Histidine	5	3	26	28	69	69
Lysine	6	0	25	28	69	72
Arginine	6	0	28	41	66	59
Threonine	5	0	32	37	63	63
Urea	27	3	17	45	56	52
$NaNO_3$	28	11	22	48	50	41
Valine	7	0	45	62	48	38
Hydroxyproline	6	62	50	24	44	14
Serine	8	7	52	45	40	48
Leucine	7	11	54	48	39	41
Isoleucine	5	24	69	28	26	48
Phenylalanine	22	7	55	48	23	45
Cysteine	8	21	81	48	11	31
Cystine	8	24	86	52	6	24
Methionine	23	38	72	45	5	17
Tyrosine	19	72	77	21	4	7
Norleucine	22	59	74	41	4	0
Tryptophan	44	52	52	45	4	3

one experiment, 24 different compounds were employed singly and in amounts calculated to provide nitrogen equivalent to that contained in the basal Pridham and Gottlieb medium. The l- and dl-forms of amino acids, purchased from Merck and Company, were used. A survey was made of the same isolates reported in TABLE 3 for carbohydrate utilization, and the data for nitrogen utilization are presented in TABLE 9. Good sources of nitrogen for growth of many isolates are glutamic acid, aspartic acid, glycine, and NH_4Cl. Tryptophan, norleucine, tyrosine, and methionine are among the least utilizable compounds. When tryptophan nutrient agar is supplemented with NH_4Cl, organisms which cannot use tryptophan are enabled to grow; hence it is concluded that this amino acid is unavailable and not appreciably toxic to many actinomycetes. Nitrogen supplied in the form of ammonium salts is used by more cultures than is nitrogen in the form of nitrate.

In other experiments, the availability of nitrogen contained in yeast nucleic acid (Eastman) and in guanine and uracil was examined. Cultures of *S. griseus*, *S. bikiniensis*, *S. noursei*, and many others can utilize nucleic acid or guanine as a sole source of nitrogen (TABLE 8). Uracil either is not used at all or is only slightly available for growth. Further studies on the growth values of purines and pyrimidines would, doubtless, yield significant data.

TABLE 10

UTILIZATION OF DIFFERENT SOURCES OF NITROGEN BY CULTURES OF STREPTOMYCES GROWN IN AGAR PLATES

Isolates	Sources of nitrogen				
	$NaNO_3$	$NaNO_2$	NH_4Cl	Glutamic acid	Peptone
C 17566a	0	0	0	++	+++
Y 75	0	0	+++	++++	+++
D 59	+++	0	+++	+++	+++
C 17558	−+−	−+−	+++	+++	+++

(Symbol 0 signifies not used; increasing use represented by ++ to ++++)

If one arranges different sources of nitrogen according to the series N_2, NO_3, NO_2, NH_3, amino acid, peptone, and protein, then it is possible to find a number of different patterns of nitrogen utilization in *Streptomyces*. The ordinary saprophytic isolates appear unable to utilize molecular nitrogen, but they can grow on different forms of fixed nitrogen; some use nitrogen of amino acids, but not nitrogen of inorganic salts; still others use ammonium or amino acids, but not nitrate or nitrite. Some of the observed types of response to different forms of nitrogen are shown in TABLE 10. Protein nitrogen may or may not be available for growth, depending upon the proteolytic activity of the cultures. Appreciable nitrite appears to be toxic to some cultures, which can metabolize nitrate and might be expected, therefore, to be able to reduce nitrite. The data shown in the lower part of TABLE 8 illustrate further the variation of responses of a few well-known species to nine nitrogenous compounds. In addition to growth, other features too, such as sporulation and pigment production, are greatly affected by the nitrogenous materials in the substrate. It is beyond the scope of this paper to elaborate further on the details. Growth responses to different forms of both nitrogen and carbon sources provide striking examples of differences in these organisms. The influence of previous cultural conditions upon enzyme activities of different isolates and the significance of these data for taxonomy will require further study.

The use of growth responses to nutrient substances for distinguishing species will be valid, if (1) the method will aid in excluding isolates which do not belong in certain known species, and (2) if such data will serve to characterize isolates as belonging within a species population. In order to test the validity of growth responses on carbon and nitrogen sources for species identity, 12 isolates, each bearing the label "*S. griseus*," were assembled from the American Type Culture Collection, Northern Regional Research Laboratory, and from Centralbureau voor Schimmelcultures. The data of TABLE 11 show that there are some differences in micromorphology in this assemblage, and that sharp differences exist with regard to utilization of rhamnose, lactose, sucrose, salicin, nitrate, leucine, and hydroxyproline.

Some properties of micromorphology and growth responses to carbon and nitrogen compounds were determined for 29 cultures labeled "*S. albus*." These cultures were received from the CBS and ATC collections. As shown

TABLE 11

UTILIZATION OF CARBON AND NITROGEN SOURCES BY DIFFERENT ISOLATES DESIGNATED "*S. griseus*"

	Source of cultures	"Y" Culture Number	Rhamnose	Lactose	Sucrose	Raffinose	Inulin	Sorbitol	Mannitol	Inositol	Salicin	Na-citrate	Na-succinate	$NaNO_3$	Leucine	Hydroxyproline	Methionine
Conidiophores long, straight, branching, or in clusters	NRRL	4	0	++	0	0	0	+	+++	0	+++	+++	+++	+++	+	+++	0
	ATC	67b	0	+	0	0	0	0	+++	0	+	+++	+++	++	+	+	0
	CBS	116d	0	++	0	0	0	0	+++	0	0	+++	+++	++	++	++	0
	CBS	116e	0	++	0	0	+	+	+++	0	+	+++	+++	++	++	++	0
	CBS	116f	0	++	0	0	0	0	+++	0	++	+++	++	++	0	++	0
	ATC	67a	+++	0	0	0	+	+	+++	0	0	++	+++	+++	+++	+	+
	CBS	116a	++	0	0	0	0	0	+++	0	+	+++	+++	+++	++	0	+
Conidiophores wavy, short, in clusters	ATC	67	0	++	+++	0	0	0	+++	0	+++	+++	++	+++	+++	0	++
	ATC	67c	0	+	0	0	0	+	+++	0	+++	++	++++	+++	+	++	0
	CBS	116	0	+	0	0	0	+	+++	0	++	+++	+++	+	++	+++	0
	CBS	116b	0	++	0	0	0	0	0	0	0	+	++	0	++	0	0
Conidiophores Spiral	CBS	116c	++	++	0	0	0	0	++	++	0	+	+	0	+++	+	++
Total positive isolates			3	10	1	0	2	5	11	1	8	12	12	10	11	9	4

(Symbol 0 signifies not used; increasing use represented by + to ++++.)

TABLE 12

SOME PROPERTIES OF 29 CULTURES OF "*S. albus*" OBTAINED FROM ATC AND CBS

Spore chains	Rhamnose	Lactose	Sucrose	Inulin	Sorbitol	Inositol	Salicin	Na Citrate	Na NO_3	Leucine	Methionine
Spirals											
17 Total.....	4	15	4	3	7	1	14	10	5	14	14
Straight											
12 Total.....	4	10	1	1	2	0	10	8	7	12	11

The number of cultures utilizing each compound is shown in each appropriate column for spiral and straight [illegible] types.

In TABLE 12, 17 of the cultures formed spiral spore chains and 12 developed straight chains. Considerable variation was observed among these isolates with regard to the availability of certain compounds, such as rhamnose, sucrose, sorbitol, citrate, and nitrate. Inositol, sucrose, and inulin were not good nutrients for most isolates, whereas lactose, salicin, leucine, and methionine were readily used by the majority, in both spiral and straight spore-chain groups. From these preliminary observations, it becomes evident that one cannot accept the identity of all named cultures without satisfactory evidence and that, within a micromorphological group of isolates, considerable variability exists in their nutritional responses.

(6) *Antibiotic Relations*

The production of about 80 different antibiotic substances is credited, thus far, to members of the genus *Streptomyces*. The spectrum or range of activity shown by the antibiotics, together with their physical and chemical properties, provides some basis for the identification of these substances in the crude beers fermented by actinomycetes. Production of antibiotics is now known to be characteristic of special strains of microorganisms rather than an attribute of species. Streptomycin is accordingly produced by strains of *S. griseus* and *S. bikiniensis*; neomycin is formed by strains of *S. fradiae* and other species; actinomycin is made by strains of *S. antibioticus*, *S. parvus*, and others. Some strains of *S. griseus* produce streptomycin, some make grisein, or streptocin, or candicidin, others produce both streptomycin and actidione, and still others are able to produce no known antibiotic substances at all. It is obvious that antibiotic production cannot provide critical indications of speciation. However, the production of special antibiotic substances still appears to be limited to only certain kinds of organisms and, when one realizes the limitations in this area, antibiosis may be taken to have some value as an adjunct to other more reliable criteria.

Another aspect of inhibitory relations in *Streptomyces* would be the possibility of differential patterns of susceptibility to antibiotic and other substances. To illustrate the concept, we have tested the inhibition of a considerable number of isolates by 12 different antibiotic substances, each used at a concentration of 1 mgm. per ml. Surface inoculations of poured plates were made with spores of different isolates, and then the antibiotics were applied in

0.1 ml. of solution to paper disks which were placed on the agar surface. After incubating 24 hours at 30° C., the inhibition in mm. was recorded for the radial zones at the edge of each paper pad. Some typical data are shown in TABLE 13. In general, the strains tested are not susceptible to moderate amounts of the antibiotic substances which they are able to produce. Actidione, in the amount used, failed to inhibit any of the 15 isolates reported in the table. The many different patterns of growth inhibition suggest that this kind of data may have some value in characterization of strains, and perhaps also species. A photograph of the antibiotic sensitivity method applied to test the similarity of response by isolates in the "*S. vinaceus*" group is shown in FIGURE 4. The similarity of *S. vinaceus*, *S. puniceus*, and other named cultures, with regard to both qualitative and quantitative inhibitions by antimicrobial substances, provides additional evidence for the probable identity of cultures in this group which have been described under different synonyms in the literature.

(7) *Phage Specificity*

Actinophages are known to attack certain strains of actinomycetes with resulting lysis and typical plaque formation. According to some investigators, actinophages may have a restricted host range similar to the well-known, limited host range of bacteriophages. It has been reported that a phage, which lysed streptomycin-producing strains of *S. griseus*, failed to attack nonproducing strains of the same species, and showed no action on another streptomycin-producing species, *S. bikiniensis*.[9] Recent studies carried on in the laboratories of Parke, Davis & Company indicate that a potent strain of actinophage lyses all streptomycin-producing strains in a group of 14 isolates, does not lyse any of the 3 grisein-forming cultures which were studied, and shows variable action against only 3 out of 12 other strains. This kind of evidence suggests that actinophages may possess properties of limited host range among strains of a species, but there is insufficient information to allow any prediction concerning their practical usefulness in actinosystematics.

(8) *Serum Diagnosis*

The success which has been achieved in bacterial taxonomy by employing immunological techniques for classifying difficult groups according to their antigenic components suggests the possibility that similar progress might be made with actinomycetes. Methods for the serological study of these organisms have been developed by Ludwig and Hutchinson,[10] whose preliminary investigations led them to conclude that agglutinin and precipitin reactions would serve as aids in identification and demonstration of relationships in the actinomycetes. Much more work is needed in this area.

(9) *Hydrolytic Reactions*

The enzymatic decomposition of various substrates provides an index to the special powers of actinomycetes. Their ability to attack proteins is readily observed in the liquefaction of gelatin and peptonization of milk. Hydrolysis

TABLE 13

INHIBITION OF DIFFERENT CULTURES OF STREPTOMYCES BY ANTIBIOTICS TESTED IN FILTER PADS. CONCENTRATION 1 mgm./ml.; ZONES IN mm. RADIUS

	Penicillin	Terramycin	Aromycin	Xanthomycin	Actidione	Actinomycin	Streptothricin	Puromycin	Neomycin	Chloromycetin	Auromycin	Streptomycin
S. vinaceus (NRLL B-1520)	0	10	0	5	0	3	0	9	6	3	11	0
S. puniceus (Pfizer 1314-5)	0	10	0	5	0	3	0	10	5	2.5	11	0
S. floridae (PD A5014)	6	11	0	11	0	11	0	12	10	2	17	0
S. californicus (ATC 3312)	8	12	0	10	0	10	0	10	7	4	16	0
D 453	0	7	0	3	0	3	0	8	4	3	12	0
S. griseus (NRRL B-1076)	5	11	3	10	0	6	4	13	7	3	12	2
S. bikiniensis (ATC 11062)	1	8	3	5	0	5	0	7	3	6	8	0
S. fradiae (ATC-10745)	5	16	8	8	0	5	0	14	2	10	17	7
S. lavendulae (NRRL B-1259)	10	15	6	10	0	10	0	15	6	16	20	12
S. venezuelae (PD A 65C)	3	10	6	5	0	6	0	11	6	0	10	5
S. aureofaciens (NRRL B-1287)	2	0	6	11	0	4	0	0	8	14	1	12
S. rimosus (NRRL 2234)	9	6	12	11	0	11	3	2	20	3	11	10
S. noursei (ATC 11455)	0	0	0	8	0	8	0	2	4	2	2	0
C 1437	8	12	7	10	0	10	10	12	7	10	14	11
D 411	0	0	0	6	0	2	0	1	2	4	2	0

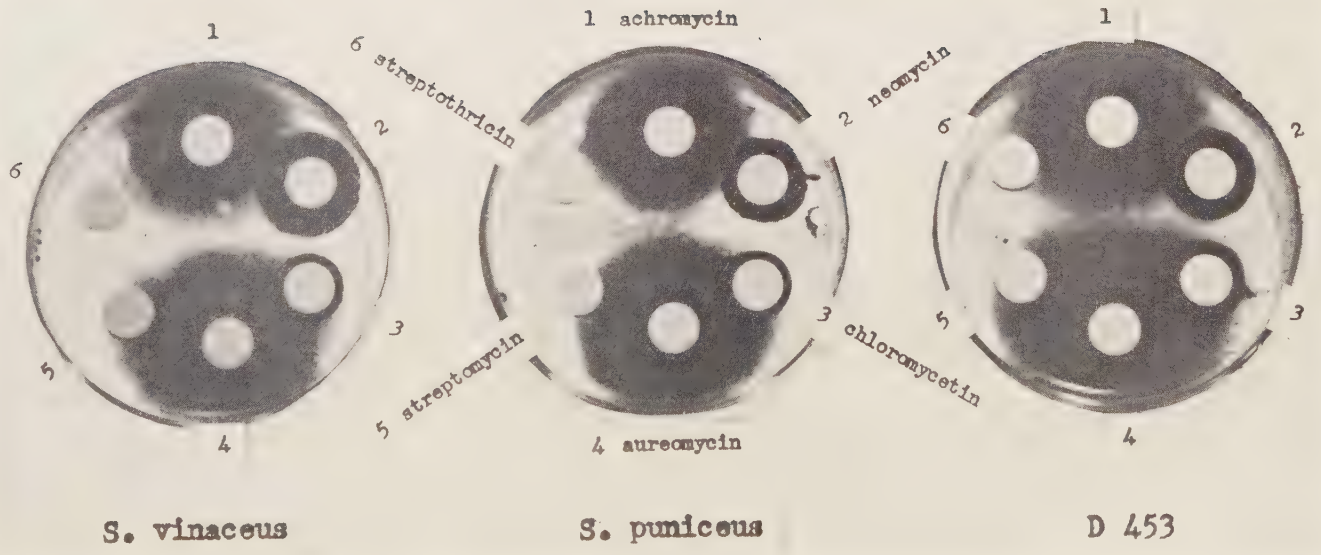

FIGURE 4. Selective antibiotic inhibition of *S. vinaceus*, *S. puniceus*, and a new soil isolate D453. Note qualitative and quantitative similarities of growth patterns in the three cultures.

of starch, inversion of sucrose, and lipolysis may supply valuable data for recognition of clones and perhaps also species. Experiments are now under way to determine the quantitative aspects of some of these hydrolytic cleavages. Sound experimental data concerning the phenomena of enzyme adaptation in these organisms are needed before the data of enzyme action can be properly evaluated in problems of speciation.

(10) *Miscellaneous*

Various other approaches toward an understanding of speciation in *Streptomyces* have been envisioned by different investigators. One of these approaches is concerned with temperature relations. The majority of the saprophytic streptomycetes are mesophilic. The genus *Thermoactinomyces*[2] thrives at elevated temperatures. In our attempts to discover psychrophiles from the Arctic circle, many isolates were grown at 10 to 15° C., but these subsequently grew well also at 25° C. Four thermophilic species of *Streptomyces* are listed in the recent book by Waksman and Lechevalier.[4] There appears to be considerable adaptation in respect to temperature tolerance.

Other factors of importance, such as hydrogen-ion concentration, oxygen relations, nitrate reduction, and the various effects of complex natural media on growth and sporulation can not be properly evaluated here. Though a few cultures can grow at pH 5.0, the best growth of most isolates takes place at a reaction near neutrality. It should be pointed out that substances present in potatoes, tomatoes, and carrots often seem to promote sporulation in isolates which form spores only sparsely, or not at all, in certain synthetic media. At the other extreme, sporulation in many isolates appears to be depressed by Difco meat-extract-peptone-agar. These isolates frequently sporulate normally in peptone agar without meat extract, or on agar containing large amounts (20 per cent) of whole cooked potato.

(11) *Conclusions*

Having presented some of the morphological and physiological criteria for speciation in the genus *Streptomyces*, a summation of viewpoint may be stated briefly. At this time, a system of convenience is needed to aid investigators in identification of isolates. Of prime importance is the use of standardized, reproducible media for this group of microorganisms, whose members respond so readily to change in the environment. A very useful general medium consists of Pridham and Gottlieb's[5] basal medium with additions of 1 per cent soluble starch, 0.5 per cent enzymatic digest of casein (N-Z-case or trypticase) and about 1.8 per cent agar. Most *Streptomyces* cultures will grow, sporulate, and produce their characteristic pigments on this inexpensive and reproducible medium. The addition of casein hydrolysate increases growth and the production of brown pigment. Many other synthetic media may be required for special use to enhance the expression of peculiar properties by different kinds of *Streptomyces*.

When working with a very large number of isolates, the primary separation of species can be approached best through the color of the aerial hyphae and

spores in mass. Some of the main types are colored white, pink, lavender, shades of grey, blue-green, yellow, tan, and intergrades of these colors. A second character, of operational value, is the observation of pigments formed in the substratal mycelium or in the medium. These pigments are commonly some shade of brown, yellow, blue, or red, and need to be evaluated in relation to pH changes and composition of the medium. A third character is the morphology of colonies, including their size, shape, and texture. Lindenbein[11] has recently set forth a good list of technical terms which may be used advantageously in describing colonies on solid and in liquid media. In addition to gross observations, it will be necessary to examine the microscopic structure of the aerial hyphae, sporophores, and spore chains, giving attention to the nature of branching and to the shape of the ultimate hyphae-forming spores. The last determinations to be made would include observations on various physiological characteristics. Included in this category are the utilization of different sources of carbon and nitrogen; of various enzymatic changes, such as hydrolytic reactions; the production of and sensitivity to various antibiotic substances; growth in nutrient solution; and any other properties which are useful.

Our present viewpoint is that, for a system of convenience, named species groups should be established, and that within these groups there may be recognized different subspecies and special strains having outstanding characters and having similar rank to the horticultural "varieties" of fruit crops. The system described by Buchanan, St. John-Brooks, and Breed[12] should meet the requirements of both theoretical investigators in colleges and of patent attorneys in industry. Recognition of strains and "cultivated varieties" (see p. 293 of reference 12) of asexually propagated clones of actinomycetes having special economic significance might open the way for including such vegetatively propagated plants in the category of patentable entities. A strain of *Streptomyces* may be defined as being an aggregate of asexually propagated organisms descending from a single isolation, possessing the characters of a named species, and differing from other strains by having some important, unique, and distinguishing properties. A species of *Streptomyces* may be defined as being a population of microorganisms which come under the description of the genus, have several fundamental characters of macroscopic and microscopic morphology which distinguish them from other species, and show accessory, discontinuous, physiological, and biochemical properties useful in formulating a reasonable working basis for those who care to study them.

Summary

The criteria for speciation in the genus *Streptomyces* are based upon characters of colonial morphology, microscopic structure of the sporophores and spore chains, production of pigments, and accessory nutritive and metabolic properties.

In order to satisfy both scientists and industrialists, it is proposed that a system of convenience be established in which relatively few species groups will be recognized and that, within these species, there will be strains having special characters and having similar rank to the horticultural "varieties" of fruit crops.

A strain of *Streptomyces* is an aggregate of asexually propagated organisms possessing the characters of a named species and differing from other strains by having some important, unique, and distinguishing properties. A species of *Streptomyces* is a population of microorganisms which satisfy the description of the genus, have multiple and fundamental characters of macroscopic and microscopic morphology which distinguish them from other species, and show accessory physiological and biochemical properties useful in an operational classification.

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VARIATION IN *STREPTOMYCES*

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The Organism

There can be no fundamental progress in our understanding of variation in *Streptomyces* until we know more about the organism itself. We need, first of all, to know whether or not the substratal and aerial mycelia are constitutionally different. This will require intensive studies in cytology and morphology, fields which can hardly be claimed to be enjoying a heyday at the present time in biology.

The proponents of a two-phase life cycle who postulate a haploid substratal mycelium and diploid aerial growth have been principally the European investigators Badian (1936), von Plotho (1940), and Klieneberger-Nobel (1947). Badian (1936) claimed that the chromatin is distributed through the hyphae as chromosomelike bodies which unite just before conidial formation, so that each conidium has a bivalent chromosome. When a conidium germinates, this bivalent chromosome undergoes two divisions, one of them a reductional division. At the same time, from one to three germ tubes arise, each of which receives one of the four daughter chromosomes. The remaining chromosome or chromosomes disintegrate.

Klieneberger-Nobel (1947), who has strongly supported the antithetic haploid-diploid life-cycle concept, believes that nuclear fusion occurs between filaments in the haploid, substratal, or primary mycelium. The fusion product is an initial cell which consists of a darkly staining nuclear body surrounded by cytoplasm and later enclosed by a cell wall. The initial cells produce the aerial or secondary mycelium.

Significantly, the point of view of Klieneberger-Nobel for a two-phase life cycle in *Streptomyces* has been applied to *Actinomyces bovis* by Morris (1951). Here he presents a haploid-diploid cycle in which there are *two* fusions and one reduction division. The fusions are described by him as follows:

"Nuclear material from the poles of cells in the 'A' phase moves to the center and fuses, during which process the cell contracts and begins to swell at one end, becoming oval or pear-shaped. If the adjacent cell remains unaltered, a drumstick appearance results. Two of these cells then conjugate, and a fusion cell is then formed in a manner reminiscent of that described by Klieneberger-Nobel (1947) for the initial cell of *Streptomyces*, and observed by the author in *Micromonospora*."

In line with the same general life-cycle point of view, Bisset (1950) has postulated nuclear fusions for *Mycobacterium* and *Corynebacterium*, and Webb *et al.* (1954) believe that in *Nocardia corallina* "nuclear process during fragmentation appears to give rise to binucleated bacillary cells. Coccoidal cells, however, are observed to be uninucleate."

Opposed to the two-phase life-cycle concept is the long accepted view that *Streptomyces* is an asexual organism in which the individual is differentiated

into a substratal portion specialized for vegetative development and an aerial portion differentiated for reproduction by conidia. An aerial filament, according to this view, may arise directly as a branch from any vegetative hypha at any point. The two are quite unlike in appearance, the aerial filament being considerably larger in diameter. Its walls are usually thicker and of a waxy consistency giving a "bloom" to the entire aerial growth.

However one regards the claims for a two-phase life-cycle in *Streptomyces*, the idea is before us and must be thoroughly tested by cytologists and morphologists. The evidence, at the present time, is altogether too meager to justify or refute the hypothesis. I strongly support Erikson (1947 and 1949) in his view that this problem is of major importance and "that in a discussion of variation, such as the frequent phenomenon of asporogenous sectors produced within a single colony, it is essential to consider the origin of the inoculum in every instance."

Let us examine some of the reported observations that relate to the two-phase life-cycle concept. First of all, are the aerial spores and substratal filaments genetically unlike? Supporting the idea is the careful work of Erikson (1948) on *Streptomyces coelicolor*, where he found that spores used as inocula frequently yielded permanent variants, whereas young vegetative filaments did not. Sister spores of the same chain gave dissimilar growths which remained so throughout 10 successive transplants. Opposed to the idea is the fact first established by Carvajal (1947) that typical spores of *Strepto-*

FIGURE 1. *Nocardia* (Proactinomyces) *ruber* grown on nitrogen-free agar at 28°C. Electron micrographs taken at 50 kv. by Professor N. M. McClung. The granules within the filaments are of unknown nature and functions.

FIGURE 2. *Streptomyces* strain S-77 of Professor Elwood Shirling. Vegetative mycelium grown 28 hours in glycerol nutrient broth. Electron micrograph of chromium shadow-cast filaments of varying diameter, some partially or completely lysed.

myces are produced by substratal mycelia in submerged culture. He showed excellent photographs of characteristic spores formed even inside of the germ tubes of conidia which were of aerial origin. Pittenger and McCoy (1953), using ultraviolet rays as a mutagenic agent, found that the mutation frequency was essentially similar for spores formed in either aerial or substratal hyphae or for spores at incipient germination.

There are few observations testing possible fusions of filaments in *Streptomyces*. The author has observed that some isolates grown from single spores or filaments on agar blocks tend strongly to have their branches grow appressed to one another. In FIGURE 3 is shown a typical instance in which the ends of filaments tend to curve, permitting the terminal portions and branches to be

firmly aligned. In older mycelia, as many as six filaments in a column have oftentimes occurred. This strong tendency for filaments to grow as compact parallel strands would permit anastomosis and possible production of heterocaryons, or perhaps nuclear fusions. Carvajal (1946) published electron photomicrographs showing evidence of fusions between germ tubes and filaments of young cultures of *Streptomyces griseus*. Pontecorvo's (1953) remarkable success in producing heterocaryons in asexual fungi by anastomoses of filaments of contrasting genotypes suggests that similar studies be attempted on actinomycetes.

Erikson (1949), however, points out that the general absence of anastomoses is one of the characteristics of the group. He says he "has watched literally thousands of growing colonies under a great variety of cultural conditions, and it has been most instructive to note the way in which, when one filament comes in contact with another, it slides over, under or around the obstruction."

Before concluding my remarks on the organism, I should like to mention the fact that I have been somewhat impressed by the heterogenous appearance of the filaments of young substratal mycelium as viewed under the electron microscope. FIGURE 2, furnished through the courtesy of Doctor Elwood Shirling, is a characteristic example. The filaments vary greatly in diameter

FIGURE 3. *Streptomyces* strain 1-Sch. of K. L. Jones. Camera lucida drawing by means of a 4-mm. objective and a 12.5 ocular of vegetative mycelium 49 hours after germination on glycerol agar block at 22°C.

and, though they were only a day old when photographed, they show small lysed portions as well as long ghost hyphae. I have not seen clear views of the interior of vegetative filaments of *Streptomyces*. McClung, however (FIGURE 1), found that, in *Nocardia*, it was possible to see internal structure in nitrogen-starved hyphae. Small dense spherical bodies of variable sizes alternated with a larger diffuse structure. Parallel studies of the same material under a light microscope confirmed the presence of the two types of granules. Neither could be identified as nuclear, since they were not seen in division, nor were Feulgen reactions consistent.

Others, *e.g.*, Carvajal (1946), Erikson (1949) and Webb *et al.* (1954), have commented on the heterogeneity of the internal organization of the aerial hyphae. Carvajal (1946) observed that spores may have one or more nuclei, and that "the number of nuclei is by no means always proportional to the size of the cell." Erikson (1949) states, "Granting that chromatinic bodies are embedded in the cytoplasm, the writer has frequently observed an irregular distribution of this material in the separate elements of sporing hyphae, as revealed by vital staining with methylene blue." Webb *et al.* (1954), who admitted that they make no attempt to follow the life cycle reported by Klieneberger-Nobel, found the "surface mycelia" of *S. griseus* to reveal cross-walls irregularly spaced along the hyphae, "with some units apparently containing several nuclei." Their one published illustration shows, very clearly, dark bodies of various sizes and shapes within the hyphae.

It is therefore possible that the internal heterogeneity of filaments and spores in *Streptomyces* leads to variability of one kind or another in subcultures grown from single spores or filaments. Growths from larger masses of inocula would tend to be more uniform.

Comments on Observations of Variability in Streptomyces

Temporary variations. Under temporary variations I shall mention, in turn, those resulting from (1) direct and immediate effect of the environment; (2) the amount or age of inoculum; (3) unknown causes apparently of an intrinsic nature which produce fluctuating variations; (4) the gradual effect of the medium leading to a change, usually a loss, of a characteristic.

Anyone beginning work on *Streptomycetes* is impressed by the profound influence of the medium and the cultural conditions on the characteristics of an isolate. Krainsky (1914) was a pioneer in championing the idea that the nature of the growth is here dependent on the substratum and that, for purposes of classification, synthetic media and standard conditions of culture are a *sine qua non*. Unfortunately, even renowned investigators do not always adhere to this principle, but employ such highly complex and ill-defined test substrates as potato plugs and yeast extract peptone agar.

I have expressed the view for some time (1940) that colony characteristics of isolates, grown on standard synthetic media under constant environmental conditions, should be given special consideration in the delineation of species. "I believe that colonies of *Streptomyces* possess convenient macroscopic characteristics of taxonomic validity that are not duplicated in larger and less definite growths on slopes and plates. These include size, shape, nature of margin,

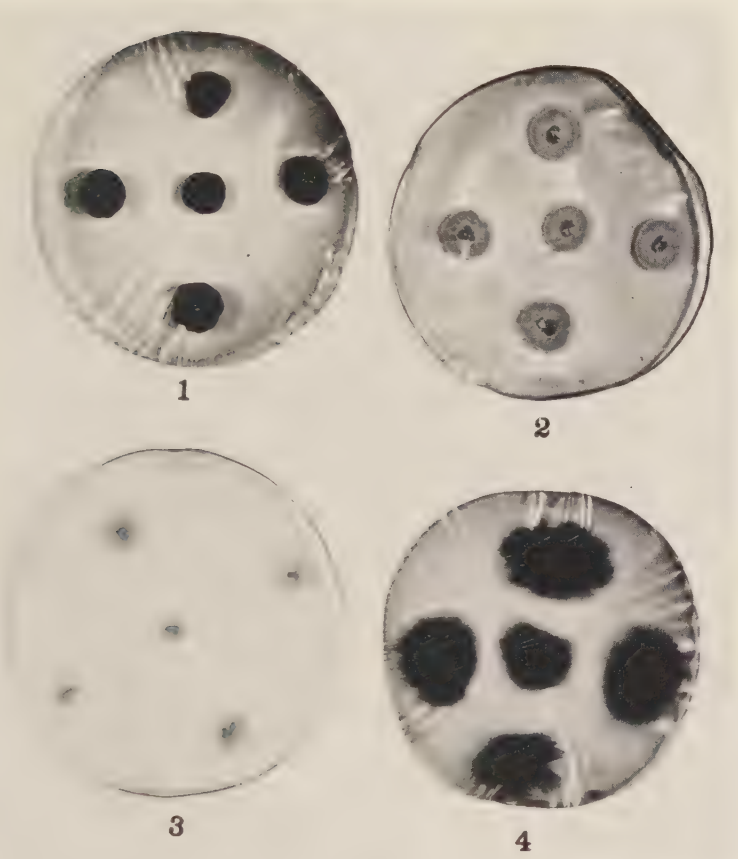

FIGURE 4. Herbarium specimens of colonies of *Streptomyces* to show diagnostic characteristics on synthetic media. The agar media were removed from the petri dishes after three weeks' growth of the colonies. The preparations were soaked in $HgCl_2$ solution to kill the organisms and to render the agar unfit for growth of molds, and they were then dried and mounted as permanent specimens.

topography position of the aerial mycelium, color of the colony and of the surrounding medium, and tendency to produce sectors" (Jones, 1950). As a practical consideration, I favor the preparation of permanent herbarium specimens of the agar films preserving the actual colonies on several synthetic media (Jones, 1950). See FIGURE 4.

It is not generally realized that the characteristics of a *Streptomyces*' isolate may be considerably altered by the medium on which it previously grew (FIGURE 5). The influence of the penultimate medium was first pointed out by Jones (1946) and Erikson (1947). This change is a temporary effect which needs further investigation on a biochemical level.

The quantity of inocula may affect pigment production and utilization of calcium malate even where vegetative mycelia alone is the inoculum (Jones,

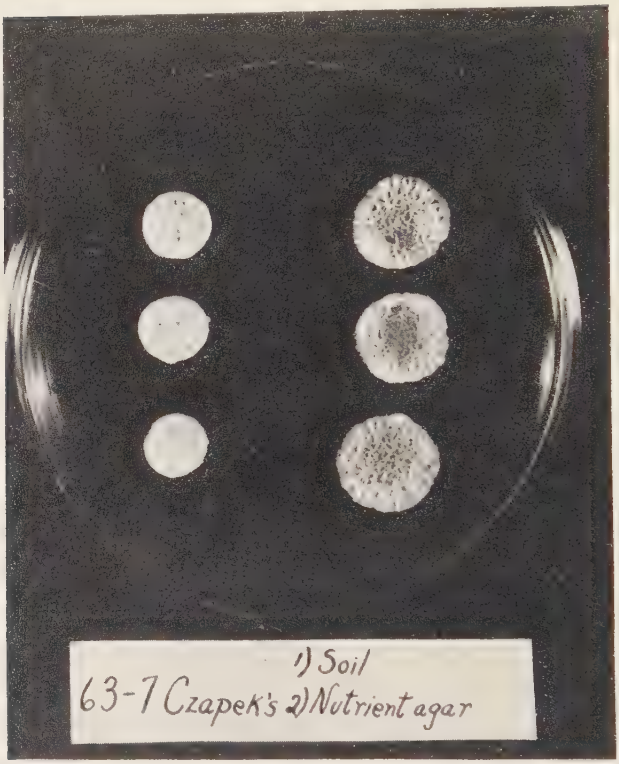

FIGURE 5. *Streptomyces* strain 63-7 of K. L. Jones, on Czapek's agar, three weeks' growth at 28°C. The effect of the penultimate medium is shown. Left row grew previously on sterilized soil and right row on nutrient agar.

1946). Erikson (1948) reported that "mass transfers of typical growth usually yielded uniform growth on plates." He found that, from a densely sporulating colony, 343 million spores were viable in each of five platinum loopfuls tested. In regard to the age of the inoculum, he stated in the same paper that stable permanent variants of *Streptomyces coelicolor* may arise from degenerate, aged vegetative mycelium, whereas parallel sets of nutrient glucose broth cultures, kept in vigorous condition by frequent subcultivation, remained essentially constant.

In certain isolates, particularly those producing diffusible pigments, a wide range of fluctuating variations may occur on each culture plate (FIGURE 6). When freshly isolated from the soil, an organism yields the entire range of variants in subcultures irrespective of the colony selected as inoculum. Fluctuating or continuous variations are apt to mislead observers to believe that the Streptomycetes are hopelessly labile. I encountered an extreme case in isolate 47-13 (1940 and 1946) which was established from a single spore but soon grew only as substratal mycelia. On Czapek's medium, it produced a greenish-black soluble pigment in some colonies. When the colonies first appeared, they were all colorless. After about six days, a pigment formed in

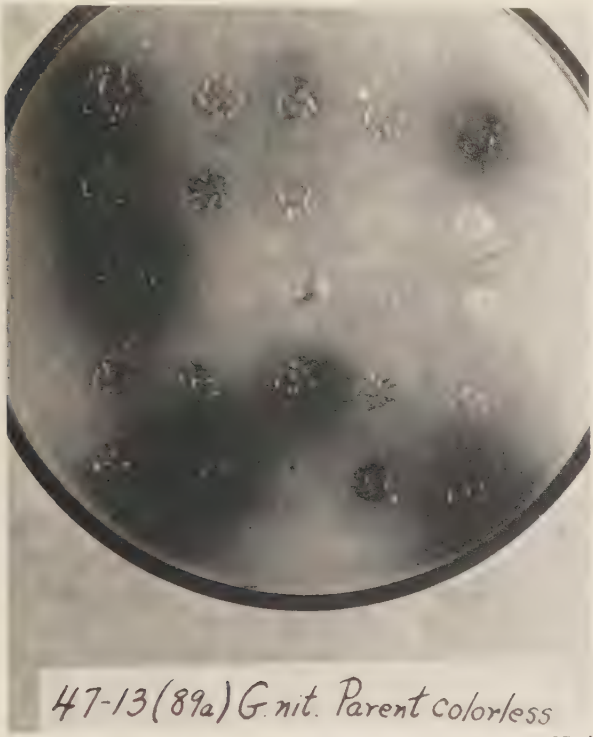

FIGURE 6. *Streptomyces* strain 47-13 of K. L. Jones grown on glycerol nitrate agar 28°C. three weeks. Fluctuating variation in pigment production is shown. The inoculum producing the colonies was colorless and asporous.

some colonies which gradually darkened and diffused. The concentration of the pigment varied, and some of the colonies remained colorless. Not only were there different intensities of the greenish-black color, but some colonies had a brown color or a mosaic of brown and greenish-black. Stanier (1942) encountered a widely fluctuating strain of *Streptomyces coelicolor* which he was culturing from spores, and he felt that the variations were probably attributable to intrinsic differences in the spores. He favored the cytological interpretations of Badian (1936) as an explanation.

Selection of colorless colonies in my asporous strain 47–13 led to a colorless line on glycerol nitrate agar after 31 months of culturing (see FIGURE 7). The result cannot be attributed solely to selection, however, as there was a gradual loss of pigmentation after prolonged cultivation on laboratory medium. Cultures transferred to soil regained their ability to produce pigmentation.

Lieske (1921) made particular mention of the gradual loss of characteristics in Actinomycetes, which seemed to him to be a widespread phenomenon in the group. In his day, it was the practice to speak of "dauermodifikation" or lasting modifications. Jollos (1921), who worked on the gradual adaptation of paramecium to the presence of arsenious acid, believed that changes occurred in the cytoplasm or in special autonomous structures of the cell. It is more

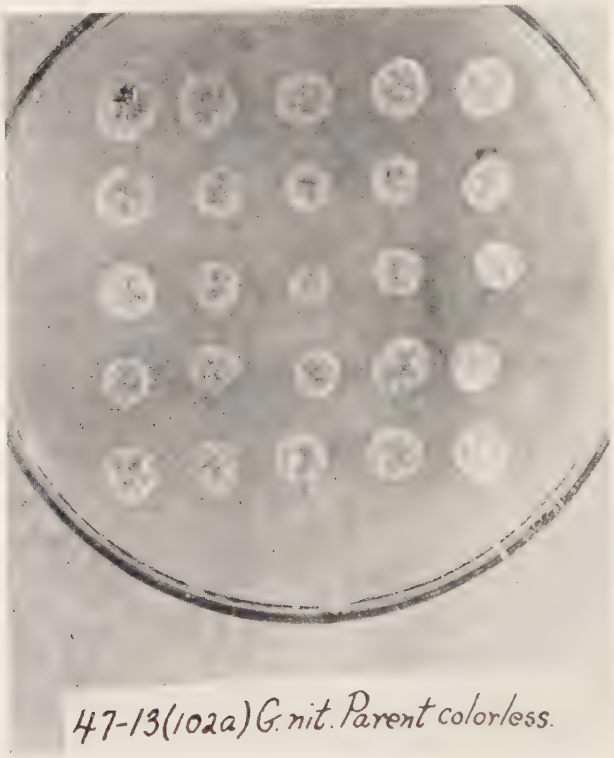

FIGURE 7. Same as FIGURE 6 after 31 months' selection for colorless ype

acceptable nowadays to approach the problem from the angle of enzyme induction and selection and mutation. We lack precise studies on the exploration of these possibilities.

Permanent variations. In considering permanent variations in *Streptomyces*, I should like to pay homage to Lieske (1921), who maintained over 100 isolates for many years, subculturing from single spores or filaments. In these isolates, he met with several permanent variations. A classical case was a 6-sectored colony in which each sector gave a different variant, the changes being in temperature requirements, odor, presence of spores, location of spores, color of spores, and diffusible pigments produced by the substratal mycelium.

Lieske was confident that the sectors he studied were not the result of any mixing of two or more kinds of inocula. He considered it possible that nuclear fusions occurred within the filaments, but doubted the actinomycetes were sexual in the sense the term was understood for higher organisms. The "colony" derived from a single spore was, to him, an individual. Sector formation suggested the vegetative sports or somatic mutations of higher plants. Erikson (1948) adeptly demonstrated that sectored colonies do not arise from two types of conidia. He mixed spores of (1) typical colored, agar liquefying

Streptomyces coelicolor with (2) colorless, nonagar liquefying variant, and grew colonies like (1) only.

Schaal (1944) clearly demonstrated that sectoring is especially associated with certain strains in the actinomycetes that produce potato scab. My observations on saprophytic soil forms confirm this. To bring out possible sector development, I suggest that the colonies be grown from single spores or at least from small amounts of inocula. The colonies should be widely spaced on the medium and allowed to develop under favorable conditions of temperature and humidity for at least a month. Skinner (Skinner, Emmons, and Tsuchiya, 1947) believes that "although variant sectors are sometimes seen, they do not seem to occur more frequently than they do in colonies of molds and bacteria of equal age." Schatz and Waksman (1945) showed that asporous variants of *Streptomyces griseus*, under certain cultural conditions, reverted to the parent sporulating strains.

It is well known that the rate of mutation can be increased by certain agents such as X radiation, ultraviolet radiation, and by nitrogen mustard ($Cl\ CH_2\ CH_2\ N$. See, for example, Savage (1949), Dulaney, Ruger, and Hlavac (1949), and Pittenger and McCoy (1953). This finding has naturally been followed with particular interest by workers intending to exploit mutants that are profitable antibiotic or vitamin producers. Medium-specific mutants have occurred, as might be expected. Apparently no correlations have been found between morphologic type and antibiotic or vitamin yield.

Pittenger and McCoy (1953) have reported on an especially interesting investigation on induced mutations in *S. griseus*. "A spore suspension was in turn exposed to five successive treatments with heavy doses of ultraviolet, each exposure followed immediately by maximum photoreactivation with visible light," as recommended for cell restoration by Kelner (1949). In this way, a relatively high yield of variants in the surviving population was observed.

The role of phage in variation of *Streptomyces* has been studied by Carvajal (1953) and by Shirling (1953). Carvajal used phage on sensitive strains of *S. griseus* to produce a variety of colony types which were strikingly different in morphological, cultural, and biochemical characteristics. He considered the varying types among the surviving population to be mutants comparable to those induced by radiation and nitrogen mustard.

Shirling found that as many as 40 per cent of the fresh isolates of *Streptomyces* from a given soil were carrying phage. Lysis was evident only if widely spaced colonies were grown. Then a small percentage were soft, yeasty, asporous colonies. One remained true to type on subculturing and yielded a high titer of phage. If this phage were added to the normal type inocula, as many as 50 per cent of the colonies formed were soft.

Normal growth occurred when phage release was low. The electron microscope revealed some well-separated ghost sections in the mycelium. Soft colonies occurred when phage production was high. Ghost sections were then more numerous and more extensive, and surviving fragments were relatively short and easily separated. Segmentation also occurred with greater fre

quency in soft types, and cell walls of ghost sections were probably more fragile. Free phage could be recovered from growths of the normal colonies but it was ineffective in increasing lysis if added to normal type inocula. Only soft colonies produced phage that was effective. Shirling considered the phage in the soft colonies to be a mutant of the less potent phage found in normal growths of his *Streptomyces* isolate.

Summary

The organism must be better understood before we can make any headway in comprehending variability in *Streptomyces*. First and foremost, an intensive cytological study is needed to give whatever direct information modern techniques can reveal on the finer structure of filaments and spores. Researches on developmental morphology will be of almost equal importance as the whole question of a two-phase life cycle must be settled one way or another. If definite nuclei exist, it may be possible to explore their genetical constitution by the production of heterocaryons through hyphal anastomoses.

In a decade hardly to be claimed as one of great distinction in morphology, it is disquieting to find life-history papers on the perplexing actinomycetes illustrated only by composite diagrams. We must publish photomicrographs and authentic drawings of particular observations. These are the basis for our ideas on nuclear behavior and sequential changes in morphology, and we must know how far each of us has progressed in acquiring raw data.

The fact of variability is so patent in *Streptomyces* that it behooves all investigators to bear it in mind. For meaningful taxonomic or genetical studies, standardized conditions must be rigidly maintained.

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USEFUL CRITERIA FOR SPECIES DIFFERENTIATION IN THE GENUS *STREPTOMYCES*

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For more than a decade, the scope of studies concerning the actinomycetes has been undergoing unprecedented expansion. This development has been the direct result of findings that selected strains of these microorganisms, principally those in the genus *Streptomyces*, possess the capacity to elaborate compounds of physiological significance. These developments have given us noteworthy contributions concerning methods for isolation of strains and for their evaluation for capacities to produce physiologically active materials. They also have given us processes for large-scale production of useful compounds. As a natural consequence, the taxonomy and phylogeny of these forms have attained added import.

Unfortunately, investigations along taxonomic lines have not kept pace with the rapidly expanding antibiotic, sterol, vitamin, and tumor-inhibiting research programs. Relatively few laboratories are pursuing any definitive program directed toward the clarification of problems associated with the identification of aerobic actinomycetes, probably on the premise that the group comprises multitudinous species and variants which defy being placed in any orderly taxonomic system. Accordingly, much valuable research material, now available in the form of cultures showing no potential economic significance, is being completely neglected. Contributions concerning the identification of new forms are usually infrequent, and they sometimes accompany the announcement of new antibiotics. In only a few instances within recent years have descriptions of new species of *Streptomyces* been presented for strains not predominantly antibiotic producers. In some instances, cultures are not being maintained properly, nor are they being deposited in acceptable culture collections with access to all concerned.

Causes of Confusion in Streptomyces Taxonomy

While the confused state of the taxonomy of these organisms has often been pointed out, the factors responsible for this condition have rarely been analyzed. Some of the factors contributing to the confusion are: (1) inadequacies of present schemes for identification; (2) lack of uniform descriptive terminology for characterization of *Streptomyces*; (3) errors in the literature; *e.g.*, citations and descriptions of experimental methods; (4) disregard of some of the reports in major publications; (5) disregard of rules of nomenclature; (6) the multitudinous media and tests employed in the characterization of *Streptomyces*; (7) lack of adequate descriptions of types and lack of type cultures; and (8) variation in methods for maintenance and identification of *Streptomyces*.

Many inadequacies exist in the present means for identification of *Streptomyces*. These deficiencies include the reliance on parasitism, on saprophytism,

and on the production of soluble pigments as major points of separation, and an absence of properly organized dichotomous keys.

The lack of uniform descriptive terminology continually plagues the investigator. Such terms as growth, pigment, *etc.*, are used indiscriminantly and are difficult to interpret. Clarification of descriptive terms and the presentation of descriptions in a clear, concise manner are needed. Such information may often be reported with little or no indication of the characters which are considered most significant. Much of the information given is, in our opinion, of little value.

Probable errors are apparent when original descriptions are compared with later studies of the same species. These errors are especially obvious in reports of characteristics directly opposed to those originally described. Under these circumstances, what are we to believe? Has the original description been emended? Has an actual error been corrected or has an error been made in interpreting the original description? In such a case we feel that the original description should be accepted. Numerous errors in citations and in explanation of experimental methods occur. Critical examination of only a few treatments will illustrate this point. Even the most modern treatments of the genus have not assembled all descriptions which appear in the literature. Synonymy of species has sometimes not been given adequate attention. Information on synonymy, in itself, would be of great value.

Still more striking has been a disregard for the established rules of nomenclature regardless of whether the Botanical or Bacteriological Code is followed. For example, *Streptothrix viridis* was described by Lombardo-Pellegrino in 1903[9] and transferred by Sanfelice to *Actinomyces* as *A. viridis* (Lombardo-Pellegrino) Sanfelice.[15] Despite this treatment, Millard and Burr[11] described, in 1926, a second form as the new species *A. viridis*. Their action can most likely be excused, since neither of the two preceding reports is cited in their paper. However, Duché,[4] who cited both Lombardo-Pellegrino and Sanfelice, described still another form as the new species, *A. viridis*. We accordingly have, in effect, three different strains all described in the literature as new species under the epithet *viridis*.

These last three factors are of considerable significance in the light of our present-day knowledge of this genus.

The problem of selection of proper diagnostic media and tests is confusing to anyone attempting to identify a *Streptomyces* for the first time because methods have not been standardized. In addition, previous lack of knowledge of these forms has led to the use of many and diverse media and tests for descriptive studies, the number of which is appalling. Table 1 lists only a few of these media and tests.

The resulting confusion can well be understood and the use of all of these substrates and tests in characterizing an isolate would follow. Unfortunately, many of these tests have been used with only a few selected strains. Little or no over-all comparative data exist. Even if the composition of the media is given and the test procedures outlined, their universal application is questioned. Aside from considerations of the prodigious amount of labor involved in their use, their reproducibility is doubtful since methods vary for the prep-

Table 1

Some Media and Tests Employed in the Characterization of Actinomycetes

Potato plugs	Asparagine agar
Carrot plugs	Glucose broth
Oatmeal agar	Asparagine-glucose agar
Dorset's egg medium	Asparagine-glucose broth
Loeffler's serum medium	Czapek's solution
Plain gelatin	Sucrose agar
Nutrient gelatin	Glycerine-asparagine agar
Ca-malate agar	Glucose-casein agar
Synthetic starch agar	Glycerin agar
Nutrient-starch agar	Serum agar
Synthetic glucose agar	Potato-dextrose agar
Nutrient-glucose agar	Coon's agar
Nutrient agar	Milk agar
Czapek's solution agar	Nitrate reduction
Cellulose media	Esculin hydrolysis
Blood agar	Starch hydrolysis
Litmus milk	Tyrosinase production
	Methylene blue reduction
	Catalase production

aration and manufacture of many components of these media. This circumstance assumes added import when one considers the sensitivity of *Streptomyces* to their nutritional environment. In our opinion, the use of completely synthetic media should play an important role in the preparation of species descriptions, supplemented, of course, with data obtained with the more generally employed substrata.

Today, numerous descriptions are inadequate. In many cases, these accounts are being relied upon in taxonomic work. However, more thorough and exacting descriptions of some of the newer species have been published, and these newer accounts may improve the situation. Fresh isolates can thus be recognized as being identical with these newly described species.

Assuming that one has arrived at a species name or, what is more often the case, a choice of any one of several names, the absence of a type culture precludes accurate comparative studies. Type cultures of some of the species already described are available, yet we know of no collection which is complete. Unlike reference material of many fungi, herbarium material is of little value and seldom, if ever, exists. Even in those cases where type cultures are available, inadequate methods of maintenance or attack by phages may have rendered them of little value. In many instances, the characteristics of these so-called type cultures do not conform with published descriptions. Improved methods for maintenance may help to clear up the problem of validity of type material.

There is a need for standardization of methods for the maintenance of type cultures. Standardization in the past has been the exception rather than the rule. In many instances, isolates are still being maintained only by the classical method of routine transfer on a single medium. With the improved methods available; *e.g.*, lyophilization and soil culture, the solution to this problem is obvious.

Of greater significance is the need for a uniformity in methods for characterizing these forms. Here, the need is quite obvious and the solution requires

only strict adherence to a uniform system. We hope that our proposals along these lines will be given considerable thought.

It is not our purpose to leave the impression that the situation is impossible. It is, rather, to point out major obstacles which, we feel, confront anyone who, without long experience, is attempting to identify a new isolate. Certainly, many of these obstacles can, in time, be overcome by careful attention to the fundamental precepts for taxonomic study.

Criteria Currently Employed in Defining Species of Streptomyces

Morphological, cultural, physiological, and ecological criteria all occupy an important position in defining species of *Streptomyces* at the present time. We should like to dwell briefly upon each of these.

Morphological criteria. There is little information concerning the comparative morphology of many species of *Streptomyces* to help in the identification of these organisms. We feel that the use of morphological criteria will, in time, serve as a sound basis for a more natural taxonomic system for these forms. Much of the information available has been obtained through use of the slide-culture technique and the slide-impression technique. Both of these have disadvantages. The slide-culture technique, because of the relatively long periods of time required for growth and sporulation of *Streptomyces*, leads to desiccation of the small quantity of substrate involved, and to atypical growth of the isolate. The slide-impression technique requires a great deal of time and experience for the preparation of suitable material. When preparations are mounted with these techniques in liquid, or are stained, the size and appearance of the morphological elements may be disrupted or changed.

Krasilnikov[7, 8] has emphasized certain morphological criteria in his key. Waksman and Henrici[17] have also established basic morphological types for the genus, but, unfortunately, there has been no strict adherence to these in subsequent characterizations.

The genus *Nocardia* should certainly be included in a discussion of the morphology of *Streptomyces*. Some *Nocardia* species would undoubtedly have been placed in *Streptomyces* if they had been observed on optimal sporulation media at the time of their isolation. Exacting study of any form which does not appear to produce aerial mycelium or spores is necessary before concluding that it is not a *Streptomyces*.

Cultural criteria. Most descriptions of species of *Streptomyces* are based upon observations of their growth habits both in and on a variety of liquid and solid substrata. Selection of these substrata has been influenced principally by past experience of bacteriologists in defining species of bacteria. Information consists for the most part in gross cultural descriptions. We feel that some of the substrata employed might well be eliminated in taxonomic procedures applied to this genus. Reliability of such characters as shape and size of colonies as employed by Krainsky[6] and by Millard and Burr for major separations of species is questionable. Likewise, we feel that the use of any liquid media under static growth conditions has little or no value. Our data is based almost entirely upon the observation of strains growing on solid substrata.

Physiological criteria. Since isolates in the genus *Streptomyces* have presented difficulties from both morphological and cultural standpoints, investigators have placed more emphasis upon their physiological characteristics in identification of species. Close adherence to bacteriological techniques has been the rule.

The chief physiological tests employed thus far include starch hydrolysis, nitrate reduction, gelatin liquefaction, action on litmus milk, and both soluble and insoluble pigment formation. Of these, pigment formation has been most heavily relied on. The reliance on soluble pigment formation has definite disadvantages since, in certain cases, conflicting results have been reported for the same species. The work of Benedict and Lindenfelser[2] emphasizes the care which must be taken in interpreting experimental results based on soluble pigment formation. They obtained soluble pigment formation with strains of *S. griseus* reported not to produce such pigment. The adherence to soluble pigment formation as a major criterion for species differentiation has the additional disadvantage that generally nonsynthetic media of indeterminate composition; *e.g.*, nutrient agar and gelatin, have been employed in determining these characteristics.

The ability of *Streptomyces* species to evince varying growth responses in the presence of selected carbon and nitrogen sources has shown some promise as an aid to species differentiation.[12] Little is known concerning the stability of this type of response over a long period of time as influenced by methods of maintenance, by variations in test condition, or, whether the results will be useful in separating a wide range of accurately defined species.

The ability of *Streptomyces* species to grow within certain temperature ranges has also been employed in aiding species differentiation. Separation of the thermophilic forms from the mesophilic forms does not appear difficult, since the former may be detected by their extremely high-temperature tolerances. As a subordinate criterion, the determination of temperature tolerances should most certainly be included in a taxonomic study of these forms.

Other physiological criteria, which at the present time occupy only a subordinate role in the differentiation of species of *Streptomyces*, include production of antibiotics, phage susceptibility, and serological characteristics. While these are of some value, the practicability of their use in routine studies is doubted.

Ecological criteria. The ecology of *Streptomyces* has assumed some significance in species separation. Thus, *S. scabies* is generally associated with potato tubers and *S. ipomoea* with sweet potato roots. Numerous different strains of *Streptomyces* have been isolated from scabby potatoes. Accordingly, identification of a strain as *S. scabies*, based on this characteristic, is questionable. For the greater number of *Streptomyces*, we consider the ecological criteria of little value.

Proposed Methods for Taxonomic Study of Streptomyces

In reporting briefly on our current studies we shall outline the procedure followed. It is our hope that its application on a universal scale may, in time, aid in clarifying species concepts within the genus.

TABLE 2
REPRESENTATIVE SOURCES OF SOME ISOLATES OF S. VIRIDOCHROMOGENES

Isolate number	Source	Comments
NRRL B-1227	ATCC 3356	Recd. as *S. viridochromogenes*
S-942	Wisconsin	Recd. as *S. viridochromogenes*
NRRL B-1511	CBS	Recd. as *S. viridochromogenes*
A-4031	New Mexico	From cotton duck
NRRL B-1132	Gold Coast, Africa	Soil sample
S-13	Sweden	Soil sample
S-68	San Salvador	Soil sample
S-109	Iowa	Soil sample
S-721	Australia	Soil sample
S-868	Illinois	Soil sample

Selection of cultures. Species concepts based upon study of a large number of similar or closely related strains from varying sources have been almost completely neglected. As a result, ranges of similarity or dissimilarity (variation) between species have not been established. Thus, as a first step, we suggest *collection from widespread sources of a considerable number of strains, tentatively related on the basis of pigmentation of sporulating cultures.*

In carrying out this first step, we selected, from among approximately 1000 fresh isolates, 39 strains designated as members of the blue-, blue-green-, or green-spored group. These strains conform, generally speaking, with the characteristics of *S. viridochromogenes* as reported in the original literature. Twenty of these isolates are quite obviously *S. viridochromogenes*.[6, 16] The remaining 19 differ from this species in a number of respects. Three related type cultures were also included in the study.

These isolates were obtained following dilution plating of many soil samples on our modification of Von Plotho's agar [glycine replaced by L (+) arginine]* and asparagine-dextrose agar.* Selection of the original 1000 isolates was limited to not more than two similarly appearing strains from a particular soil sample following comparison of all isolates in pure culture on a good sporulation medium.

TABLE 2 indicates the widespread occurrence of *S. viridochromogenes* as based on our isolation data.

The remaining 19 isolates form blue-, blue-green-, or green-pigmented spores in mass, but they differ from *S. viridochromogenes* in a number of respects. These isolates were included in our study for comparative purposes. Their exact taxonomic position is unknown at present but will be clarified when a sufficient number has been obtained for adequate study.

Morphological criteria. We believe that, when species of *Streptomyces* are defined sufficiently for accurate recognition, the system of identification will be

* Prepared as follows:

	glucose	10.0 gm.
L(−)	asparagine	0.5 gm.
	$K_2HPO_4 \cdot 3H_2O$	0.5 gm.
	beef extract	2.0 gm.
	agar	17.0 gm.
	H_2O (tap)	1000.0 ml.

Sterilize 15 min. at 121.5°C. Do not adjust pH.

based upon a combination of morphological and physiological characteristics. In this respect, we suggest as a second step *determination of the morphology of strains growing on a number of substrata selected by virtue of their ability to induce the formation of aerial mycelia and spores.* We obtained such information by two simple steps: (1) by a preliminary determination of the substrata most suitable for sporulation; and (2) by observations of sporulating cultures with the microscope. Isolates were cultivated on agar slants of the following media: Bennett's agar,[5] Emerson's agar,[4] asparagine-dextrose agar, Czapek's solution agar,[11] starch agar,[16] potato-dextrose agar,* yeast-extract agar,† Carvajal's oatmeal agar,‡ and corn steep liquor agar.[14] Cultivation on these media for 10 to 14 days at 28° to 30°C. usually indicated the substrate most suitable for sporulation. Precise information concerning the morphology of the strains was obtained by streaking spores upon the surface of several of the most suitable substrata in Petri plates, incubating for 10 to 14 days at 28° to 30°C., and examining the culture periodically with 100× and 440× magnification. FIGURE 1 illustrates the manner in which species of *Streptomyces* were cultured for this examination.

Each isolate was routinely studied on at least three of the optimal sporulation substrata. Employing this general technique with hundreds of cultures both named and unnamed, one of us (C. W. H.) has concluded that the morphology of a particular strain of *Streptomyces* is essentially the same on any medium where sporulation occurs. There are five basic morphological types in the genus. These types may in turn be divided into a number of subtypes. Presented in Ainsworth and Bisby's terminology[1] these are:

(1) Conidiophores not restricted in length, bearing fertile branches in verticils (whorls), with conidia more or less strongly attached.
  (a) Fertile branches in simple verticils, branches not ending in spirals.
  (b) Fertile branches in simple verticils, branches ending in spirals.
  (c) Fertile branches with compound verticils, branches not ending in spirals.

(2) Conidiophores with branches all straight, never ending in spirals; verticils absent.

* Prepared as follows: three components are prepared separately.

1. $MgSO_4 \cdot 7H_2O$	0.2 gm.
$CaCO_3$	0.2 gm.
glucose	20.0 gm.
H_2O (distilled)	100.0 ml.
2. agar	15.0 gm.
H_2O (tap)	400.0 ml.
3. peeled white potatoes	200.0 gm.
H_2O (tap)	500.0 ml.

Boil for 10 min., filter through 2 layers of cheesecloth.
Bring each of these components to 100°C., mix, tube, and sterilize for 15 min. at 121.5°C. Do not adjust pH.

† Prepared as follows:

yeast extract	4.0 gm.
malt extract	10.0 gm.
glucose	4.0 gm.
agar	24.0 gm.
H_2O (distilled)	1000.0 ml.

Sterilize for 15 min. at 121.5°C. Do not adjust pH.

‡ Prepared as follows:

rolled oats	65.0 gm.
H_2O (distilled)	1000.0 ml.

Cook to thin gruel in double boiler, filter through several layers of cheesecloth, and bring up to 1000 ml. while still hot. Add 2 percent agar and sterilize for 15 min. at 121.5°C. Do not adjust pH.

FIGURE 1
Plate culture for examination of morphology of *Streptomyces*: Czapek's solution agar, 14 days, 28°–30°C. *S. viridochromogenes*.

(3) Conidiophores predominantly in tufts, never verticillate; outline of branches flexuous and irregular.

(4) Conidiophores with branches ending in spirals; verticils absent; conidiophores either as long stalks bearing very short branches, or as short stalks bearing branches irregularly.
   (a) Branches ending in open spirals with many turns.
   (b) Branches ending in closed spirals with few turns, thus appearing as tight knots.

(5) Conidiophores with long and straight branches with spirals of a large diameter at their ends; spirals usually with only a few turns; never verticillate.

No strains have been observed in which the conidiophores are unbranched, except where they are growing under unfavorable conditions or in degenerated type cultures.

Because of the morphological specialization occurring with *Streptomyces* of the whorled type, cultures are floccose in texture rather than granular or pow-

dery. When teased with a needle, the mycelium is adherent, rolls into balls, and is difficult to disperse evenly upon the surface of nutrient substrata. Cultures apparently spread more rapidly over the surface of solid media than do those of other morphological types, and all prefer Carvajal's oatmeal agar or starch agar for abundant sporulation.

In recording results of our morphological studies, we regularly indicated morphological type, maximum length of the conidiophores, diameter of the spirals, approximate numbers of turns in the spirals, and shape of conidia. Conidial shape has been emphasized by Krasilnikov but this feature needs further study before it can be useful. Likewise, more knowledge concerning the manner in which conidia are delimited would be of value. The minute dimensions of the conidia and diameters of conidiophores and substrate mycelia preclude practical application of these measurements in accurately delimiting species. Any peculiar morphological feature; *e.g.*, presence of chlamydospores, was recorded when it occurred.

In applying the foregoing technique to our present study, all of the isolates in the blue-, blue-green-, or green-spored group presented a certain well-defined morphological pattern. All are characterized by the formation of branches ending in open spirals with many turns; *i.e.*, morphological type 4a. Table 3 presents representative results and indicates our manner of recording the data.

Cultural criteria, determination of spore color. We next considered the cultural criteria or gross morphological aspects of growing cultures. For the most part, this step consisted of determining the ability of strains to form pigment and noting the texture of growing cultures. As a third step we accordingly suggest *determination of the spore color of strains following cultivation on optimal sporulation substrata.* The texture of colonies was noted but primary emphasis was placed on spore color.

The ability of *Streptomyces* to form soluble pigments has usually been considered of major importance for species differentiation in other treatments. We believe that species differentiation can be given more substance by regarding this as a character subordinate to spore color. To define spore color more

Table 3

Representative Results Obtained in Morphological Studies of Species of Streptomyces

Strain	Substrate	Morphological group	Max. length of conidiophores μ	Diameter of spirals μ	Approximate no. of turns in spirals	Shape of conidia	Other; e.g., chlamydospores
S. viridochromogenes NRRL B-1227							
10–14 days, 28°–30°C.	Czapek's*	4a	250	5	5	globose to cylindrical	—
10–14 days, 28°–30°C.	ADA†	4a	300	5	5–6	globose to oval	—

* Czapek's—Czapek's solution agar
† ADA—asparagine-dextrose agar

adequately we mean the *en masse* color of the surface of a particular isolate when spores have been formed and do not mean the color of the spores when observed with the microscope.

Any culture designated as white by this method is always regarded with suspicion until abundant spores have been produced and the strain remains white. The importance of studying all forms on a number of substrata prior to making any decision as to the morphology and spore color of a particular isolate cannot be overemphasized. Color characteristics should, of course, be determined at maturity and the substrate and conditions employed should be reported. In certain forms, it is important to observe changes in color after maturity since these are quite marked; *e.g.*, the fading of the lavender spore color of *S. lavendulae* to gray upon aging of the culture.

Once the true spore color has been determined through use of a standard color manual, *e.g.*, Ridgway,[3] the isolates can be placed in one of five more or less precisely defined color groups as given below.

Spore Color Groups

(1) Lavender-, red-, or pink-spored isolates
(2) Blue-, blue-green-, or green-spored isolates
(3) Yellow-spored isolates
(4) White-spored isolates
(5) Gray-, gray-brown-, olive-gray-, or blackish-gray-spored isolates

We propose, at this point, a tentative key to the major groups of *Streptomyces* and some minor subdivisions. We believe that this key enables a more natural grouping of the species. While, in some respects, it resembles that of Krasilnikov,[7] we believe that with more emphasis placed on morphology, it will lead to an increased understanding of the relationships of strains and will result in the selection of better defined groups for more precise cultural and physiological studies in delimiting species. In certain other respects, the key resembles that of Waksman and Henrici, although we have eliminated considerations of parasitism, saprophytism, and soluble pigment formation which, in our opinion, tend to confuse rather than to aid the present-day investigator.

KEY TO THE MAJOR GROUPS OF STREPTOMYCES

I. Branches of conidiophores in verticils (whorls); conidia not readily separating; colonies floccose and spore color white, pink, lavender, or tan..... .....*S. reticuli* group (a)
(This group may be further subdivided as shown below.)
(a) Whorled branches simple..... (b)
(b) Branches straight.
(b) Branches spiraled.
(a) Whorled branches compound, that is, again branched..... (c)
(c) Ends of ultimate branches straight.
(c) Ends of ultimate branches spiraled.
I. Branches of conidiophores not borne in verticils (whorls); conidia readily separating when mounted in water; spore color often not pink, white, lavender, or tan..... II
II. Spore color in shades of blue, blue green, or green; always with spirals
S. viridochromogenes group
II. Spore color shades not blue, blue-green, or green; spirals may be present or absent.. III
III. Conidiophores and branches flexuous, irregular in outline but spirals never present; tufts often present; surface of colony mealy in texture on good sporulation media; spore color greenish tan or tan, never white.....*S. griseus* group.
.. IV
III. Not as above

IV. Spore color lavender, red, pink, or nearly tan: branches never flexuous
S. lavendulae group.

(This group may be subdivided into three groups.)
(a) With spirals on short branches.
(a) With spirals at tips of long straight branches.
(a) Without spirals and with straight branches.

IV. Not as above V
V. Spore color white or nearly so ..... *S. albus* group
V. Spore color never white VI
VI. Spore color in definite shades of yellow ..... *S. parvus* group
VI. Spore color in shades of gray, gray-brown, olive-gray or blackish-gray
Gray-spored group.

(This large group may be subdivided into two groups.)
(a) Spirals present.
(a) Spirals never present.

This key is intended primarily for the placement of isolates in well-defined morphological and spore color groups and not for species differentiation. Species differentiation is based principally on physiological criteria supported by certain cultural criteria.

Cultural criteria, characteristics other than spore color. Solid substrata were used almost entirely for determining the cultural characteristics of our isolates. Because of the importance of reproducibility of nutrient substrata, primary emphasis was placed upon completely synthetic media such as Czapek's solution agar and calcium-malate agar.[16] As valuable supplementary substrata of indeterminate composition, starch agar, Bennett's agar, Emerson's agar, asparagine-dextrose agar, potato-dextrose agar, and potato plugs were used. Accordingly, as a fourth step we suggest *exhaustive study of the cultural characteristics of the strains employing Czapek's solution agar, calcium-malate agar, starch agar, Bennett's agar, Emerson's agar, asparagine-dextrose agar, potato-dextrose agar, and potato plugs.*

The spore color, the presence or absence of surface exudate (if an exudate occurred the color was recorded), the occurrence or nonoccurrence of deliquescence, the reverse color, the occurrence or nonoccurrence of soluble pigment (if soluble pigment occurred the color was given), texture and height of the aerial mycelium, any distinct changes of spore color with age, and any other peculiar characteristic of the isolates were all recorded in a definite and orderly fashion as illustrated in TABLE 4. In most instances, the aerial mycelium *per se* is white. In a few cases, however, it takes on a different color which is noted.

TABLE 4
REPRESENTATIVE RESULTS OBTAINED IN CULTURAL STUDIES OF SPECIES OF STREPTOMYCES

Strain	Substrate	Spore colors	Surface exudate	Deliquescence	Color of reverse	Soluble pigment
S. viridochromogenes NRRL B-1227 10–14 days, 28°–30°C.	Czapek's	light olive gray (Ridg. Pl. LI.)	—	—	greenish	—
S-635 10–14 days, 28°–30°C.	Czapek's	—	—	—	reddish brown	dull reddish brown

The results obtained with the 20 isolates of *S. viridochromogenes* agreed in every particular, while those obtained with isolates such as S-635 differed from this species in at least several characteristics. However, all have the common group characteristics; *i.e.*, blue-, blue-green-, or green-spore color and morphological type 4a.

Physiological criteria. Certain physiological tests gave us much useful information which will doubtless be of value in species differentiation as our studies progress. Therefore, as a fifth step we suggest *exhaustive study of physiological characteristics of isolates including: action on gelatin, action on peptone-iron agar; effect of pH on soluble pigment; effect of temperature on formation of aerial mycelium, spores, and soluble pigment; utilization of carbon and nitrogen compounds under defined conditions; and determination of antibiotic activity.*

Other tests of a supplementary nature include starch hydrolysis, reduction of nitrate, and action on litmus milk. At the moment, we do not feel that these tests are of much significance. The use of litmus milk has questionable value, since it is a liquid substrate incubated under static conditions. We have encountered considerable difficulty in interpreting results obtained with this substrate in the past.

Results obtained with isolates on plain gelatin were recorded as illustrated in TABLE 5.

Significant differences between *S. viridochromogenes* and the remainder of the blue-, blue-green-, or green-spored isolates were noted on this substrate.

The use of peptone-iron agar also proved of value, as certain isolates had the capacity to blacken this medium while others caused no apparent color change. The contrast between positive and negative reactions with this test was quite marked.

The effect of pH on soluble-pigment formation and spore color was studied routinely by cultivation of isolates on asparagine-dextrose agar at pH 5.5 and at pH 8.5. With *S. viridochromogenes*, pH exhibited no apparent effect on pigmentation other than an alteration in its intensity. This test, however, might be of real supplementary value in developing species concepts for *S. coelicolor* and *S. fulvissimus*.

TABLE 5

CHARACTERISTICS OF BLUE-, BLUE-GREEN-, OR GREEN-SPORED STREPTOMYCES ON PLAIN GELATIN (14 DAYS AT 28°C.)

	Isolate number	Soluble pigment	Aerial mycelium	Liquefaction (average mm.)
S. viridochromogenes	B-1227	brown	+	5
	S-13	brown	+	5
	S-68	brown	+	3
	S-109	brown	+	6.5
	S-721	brown	+	7.5
Other blue-, blue-green-, or green-spored isolates	S-70	reddish brown	−	10
	S-111	brown	+?	0
	S-160	reddish brown	−	7
	S-635	purple	−	2
	S-700	—	−	4.5

TABLE 6

EFFECT OF TEMPERATURE ON THE BLUE-, BLUE-GREEN-, OR GREEN-SPORED STREPTOMYCES GROWN ON ASPARAGINE-DEXTROSE AGAR FOR 14 DAYS

	Isolate number	Formation of aerial-mycelium				Production of spores				Soluble pigment			
		24	32	36	41	24	32	36	41	24	32	36	41
S. viridochromogenes	B-1227	+	+	+	+	+	+	+	+	+	+	+	+
	S-13	∓	∓	∓	+	+	+	+	+	+	+	+	+
	S-68	+	+	+	+	+	+	+	+	+	+	+	+
	S-109	+	+	+	+	+	+	+	+	+	+	+	+
Other blue-, blue-green-, or green-spored isolates.	S-70	+	+	+	−	+	+	+	−	+	+	−	−
	S-111	+	+	+	+	−	+	+	+	+	+	−	−
	S-160	+	+	+	−	+	+	+	−	+	+	+	−
	S-635	+	+	+	+	−	−	+?	+	+	+	+	−

Temperature studies also played a part in our techniques. Isolates were cultivated on selected media at 24°, 32°, 36°, and 41°C. After an appropriate interval (14 days), the growth characteristics were recorded as shown in TABLE 6.

Isolates which grow well at 41°C. could be studied subsequently at higher temperature levels.

Other physiological criteria which we employed as an aid in establishing species concepts are the abilities of strains to utilize selected carbon and nitrogen sources in synthetic media. It was found that all *S. viridochromogenes* isolates exhibited the same carbon utilization pattern with minor exceptions. Moreover, all of the 42 isolates in our blue-, blue-green-, or green-spored morphological type 4a group exhibited a definite group pattern. When this pattern was compared with patterns obtained with other groups wherein relatively few strains were examined, definite differences were observed. Representative results obtained in our studies are illustrated in TABLE 7.

Nitrogen utilization studies would be run in the same manner. This phase of our work has not yet been completed. Utilization information correlated well with other tests employed. Since the isolates studied were obtained from a wide geographical area, the results appear to constitute additional proof of the natural physiological relationships within the group.

To supplement further information for developing a species concept, although we are aware of the apparent objections, the antibiotic spectrum of each isolate was determined. With the group presently under study, correlation with other characters was noted. Thus, none of the isolates of *S. viridochromogenes* grown on the surface of M1 or M7 media for four days were active against 12 test microorganisms. On the other hand, the remaining isolates showed varying antibiotic activity as illustrated in TABLE 8.

It was most fortunate that earlier data made *S. viridochromogenes* capable of easy recognition. Moreover, available type cultures had apparently maintained their stability. Since fresh isolates in this group agreed, for the most part, with the older species descriptions, we propose to define the species more

TABLE 7

CARBON UTILIZATION PATTERNS OF REPRESENTATIVE STREPTOMYCES ON PRIDHAM AND GOTTLIEB'S MEDIUM[12] (10 DAYS AT 28°–30°C.)

	Isolate number	Sucrose	Rhamnose	Lactose	Melibiose	Raffinose	D-Mannitol
S. viridochromogenes	B-1227	(−)	+	+	+	+	+
	S-13	(−)	+	+	+	+	+
	S-68	(−)	+	+	+	(−)	+
	S-109	(−)	+	+	+	⊕	+
Other blue-, blue-green-, or green-spored isolates	S-70	+	+	+	+	+	+
	S-111	+	+	+	+	+	+
	S-160	+	+	+	+	+	+
	S-635	+	⊕	+	+	+	+
S. aureofaciens	2209	+	(−)	⊕	(−)	(−)	(−)
	B-1286	+	−	(−)	−	(−)	(−)
	B-1287	+	−	⊕	−	(−)	(−)
	B-1288	+	(−)	⊕	−	(−)	(−)

\+ Positive utilization
⊕ Poor to fair growth
(−) Faint growth, probably no utilization
− No growth
All isolates − on control
All isolates + on glucose

TABLE 8

REPRESENTATIVE ANTIBIOTIC SPECTRA* OF BLUE-, BLUE-GREEN-, OR GREEN-SPORED STREPTOMYCES

NRRL strain		Substrate†	*B. subtilis* B-558	*B. subtilis*§ B-978	*A. aerogenes* B-199	*C. albicans* Y-477	Bodenheimer‡ B-962	*M. smegmatis* B-313	*E. coli* B-766	*M. tuberculosis* B-692	*Br. bronchiseptica* B-146	*Sarcina* B-1018	*Ps. aeruginosa* B-23	*P. vulgaris* B-117
S. viridochromogenes	B-1227	M1	0	0	0	0	0	0	0	0	0	0	0	0
		M7	0	0	0	0	0	0	0	0	0	0	0	0
	S-13	M1	0	0	0	0	0	0	0	0	0	0	0	0
		M7	0	0	0	0	0	0	0	0	0	0	0	0
Other blue-, blue-green-, or green-spored isolates	S-111	M1	12	10	0	13	0	13	0	10	0	15	0	0
		M7	0	0	0	0	0	0	0	0	0	0	0	0
	S-635	M1	5	5	0	0	0	12	0	0	0	7	0	0
		M7	4	0	0	0	0	15	0	0	0	0	0	0

* Mm. inhibition.
† Special media for antibiotic spectra.
‡ Streptomycin resistant.
§ Streptothricin resistant.
Streptomyces cultivated 4 days at 28°–30°C., test organisms cultivated 2 days at 37°C.

precisely in keeping with our advances in knowledge and with new techniques employed.

If, upon completion of the above steps, a worker feels that his strain does not agree with previously recognized forms and merits description as a new

species, we suggest that he make a *thorough search of the literature; adhere to the rules of nomenclature; deposit a typical isolate in at least two reliable type culture collections; and preserve typical strains by lyophilization, by soil culture, and by routine transfer on optimal sporulation media.*

Summary

Taxonomic methods employed in species characterization of *Streptomyces* have been reviewed and evaluated. Need for uniformity in methods of study and reporting of data has been emphasized. Methods which appear to be of most importance in defining a species have been enumerated.

The authors suggest a method for study of species of *Streptomyces* as based upon results obtained in studies of isolates related to *S. viridochromogenes.* The method is commented upon and presented as outlined: (1) the collection, from widespread sources, of a considerable number of closely allied strains related on the basis of pigmentation of sporulating cultures; (2) a comprehensive study of morphology on a number of media selected by virtue of their ability to induce formation of aerial mycelium and spores; (3) the determination of the spore color on optimal substrata; (4) an exhaustive study of cultural characteristics on selected substrata; (5) an exhaustive study of physiological characteristics on selected substrata; and (6) thorough and accurate coverage of the earlier literature, strict adherence to the rules of nomenclature, deposit of a typical isolate in at least two reliable type culture collections, and preservation of typical strains by approved procedures.

A key, based on morphology and spore color, is proposed for separation of the major groups in the genus. The use of physiological criteria in more precise delimitation of species is stressed.

Excellent correlation between microscopic characteristics, gross cultural appearance, and physiological activity was noted with the isolates studied.

Acknowledgment

We should like to acknowledge the assistance of Patricia M. Anderson in obtaining some of the data and of Dorothy I. Fennell of the Northern Utilization Research Branch, and Doctor David Gottlieb of the Department of Horticulture, University of Illinois, for reviewing the manuscript.

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EKTACHROME TRANSPARENCIES AS AIDS IN ACTINOMYCETE CLASSIFICATION*

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The identification of any culture in any group of organisms is a responsibility that cannot be considered lightly. In the group of organisms known as the Actinomycetes, there exists great taxonomic confusion. This condition is fostered by a serious lack of standardized conditions for culture comparison.

I have to offer, to those interested in actinomycete taxonomy, a method of culture comparison that I believe excels all others; namely, a collection of 4 × 5 Ektachrome transparencies, each transparency representing one species on six diagnostic media. Sporulating and reverse surfaces are shown.

Ektachrome transparencies make possible a visual comparison of cultures, grown under standardized conditions—a decided advantage over nonstandardized printed comparisons. The ideal situation would be to have all cultures, treated under identical conditions, available for comparison at one time. The work factor alone makes this prohibitive. It is possible to treat cultures in a standardized manner and photograph them to form a ready reference library. This approach should open new vistas to all interested in the actinomycete problem.

In the preparation of color transparencies, Ektachrome was chosen first, because we found it to have a truer color; second, because it comes in cut film sizes; third, because it may be processed the day the material is photographed. If a retake is necessary, less time is lost.

The slant preparation procedure is as follows: an inoculating needle loopful of soil, from the soil stock in which the culture is maintained, is inoculated into 100 ml. of broth† in a 500-ml. Erlenmeyer flask. The flask is incubated at 28° C. on a reciprocal shaker making 94 strokes min. After 48 hours incubation, the shake flask material is Waring blended one minute; then 0.2 ml. of the blended inoculum is pipetted on the surface of each of six different duplicate agar slants. The slants are incubated seven days at 28° C. At the end of this time, an Ektachrome transparency is made.‡ The media used are Bennett's,[1] Czapek's sucrose,[2] maltose-tryptone,§ peptone-iron,[3] Waksman's tyrosine,[2] and casein starch.¶ All media (broth and slant) are autoclaved for 15 minutes at 15 lbs. pressure and 120° C.

* The work described in this paper has been made possible through the kind cooperation of many Upjohn personnel and, in particular, through the interest of G. M. Savage and N. A. Drake.

† 0.5% yeast extract, 0.3% tryptone, distilled water.[4]

‡ Recommended Eastman process used.

§ Composition: gram/liter distilled water

Maltose	10.0
Bacto-tryptone	5.0
K_2HPO_4	0.5
NaCl	0.5
$FeSO_4$	0.1
Agar	20.0

¶ Composition: gram/liter distilled water

Sodium caseinate	2.0
Soluble starch	1.0
K_2HPO_4	0.2
$MgSO_4 \cdot 7H_2O$	0.2
$FeSO_4 \cdot 7H_2O$	Trace
Agar	20.0 (add after heating)

As soon as interest develops in a culture, it can be put on these six media and compared with identified species in the Ektachrome library. Various combinations of pigmentation and sporulation on the media used enable one to develop a highly workable key. For example, the *Streptomyces* species processed to date fall into two distinct groups on the basis of H_2S darkening or lack of it on the peptone-iron agar.

It is important to know as soon as possible if an antibiotic-producing actinomycete is new or identified. Early comparison of Ektachromes eliminates 80 to 95 per cent of the known actinomycete species. Supplementary culture comparison is needed for the final 5 to 20 per cent. Examples of the utility of this system are as follows:

(1) *Determination of Identity of Cultures Producing the Same Antibiotic*

(a) *Cultures of the same species.* Some cultures isolated in our antibiotics screening program showed antibiotic properties similar to those of Waksman's *S. fradiae* 3535, which produces neomycin. The growth, on the six media cited, was identical for the knowns and the unknowns. The antibiotic produced by the unknowns was isolated and purified and found to be neomycin.

(b) *Cultures of different species.* Two identified streptomycin-producing cultures: Waksman's *S. griseus* * 4 and *S. bikiniensis* NRRL B-1049 have widely differing cultural characteristics. They are sharply differentiated on the peptone-iron agar. *S. griseus* does not fall into the H_2S darkening group. It has cream-colored to pink sporulation on Bennett's and maltose tryptone agars with honey-colored pigmentation. *S. bikiniensis* falls into the H_2S darkening group. It is characterized by heavy gray-blue sporulation and dark brown pigmentation on Bennett's and maltose tryptone agars.

(2) *Detection of Similarity and or Identity in Species Described Years Apart and Handled under Different Conditions*

S. microflavus ATCC 3332 and *S. willmorei* ATCC 6867 appear strikingly similar on the elective culture media employed. Such similarities may be overlooked by the individual comparing cultures by keys which rely on word descriptions with color definitions based on the increasingly inaccessible Ridgeway publication of 1912.

In the short time we have used Ektachrome transparencies in culture comparison and identification work the results have been extremely satisfactory.

This method of culture comparison could be of great advantage, to all concerned with actinomycete taxonomy, if it could be more widespread. Strict adherence to specified conditions of inoculation, medium formula and preparation, incubation, temperature, and period of incubation would be necessary.

It is possible to visualize a central comparison service or transparency exchange system which would: (1) permit a rapid visual comparison of cultures without the need of maintaining many cultures; and (2) eliminate much of the confusion caused now by comparison with written keys which tend to call a rose by many names.

Confirmation of this early indication of identity or newness could then be

followed by the carbon assimilation technique of Pridham and Gottlieb and by other well-defined diagnostic tests.

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INDUCED MUTATION AND STRAIN SELECTION IN SOME INDUSTRIALLY IMPORTANT MICROORGANISMS

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Variations in imperfect fungi pose problems in taxonomy that have been with us since study of these organisms began and that will concern us as long as such study continues. Solution of these problems is of paramount importance to students of fungus physiology who are now probing into the biochemistry of fungi whose taxonomy and nomenclature are obscure, to say the least. Fortunately, we now have workable systems in the common genera, *Aspergillus*[4] and *Penicillium*.[5] The genus *Fusarium* no longer seems so ponderous and unwieldy owing to the work of Snyder and Hansen.[26, 27, 28] Neergaard's treatment of *Alternaria* is an excellent foundation for further work with this common genus.[17] One group that is now in dire need of a rational approach is the Actinomycetes. The genus *Streptomyces*, in particular, would seem to be a promising mistress to some young mycologist willing to devote to her the best years of his life. Such a union should be quite fruitful. It is not the object of this paper, however, to discuss the problems and pitfalls involved in studies of variability as related to taxonomy and natural relationships; but a plea is made here for an experimental approach to such problems. Continuing descriptions of taxons without prior experimentation and without a full knowledge of the whole biology of the organisms in question, where this factor can be explored, will lead only to further confusion.

The inherent variability that often has been so perplexing to many investigators offers hope to others. Variation in morphological or phenotypical characters are mere expressions of differences and variations in the biochemistry of the organisms. These biochemical activities are under genetic control, and it is now clear that this variability can be increased by various means. It is the use of natural and induced variation in some organisms of industrial importance that will be the subject of this paper.

When one is attempting to increase the yield of a product elaborated by an organism, two general pathways are open. We are not concerned here with improvements in purification and recovery. The first of these involves study and improvements of the fermentation environment. The second lies in obtaining superior producing organisms. In the first pathway are included all factors external to the organism; *e.g.*, aeration, agitation, pH, temperature, precursors, nutrients, *etc.* Once the combination of conditions optimum for maximum production is obtained, only the second pathway is left as a means of increasing production. That is, an organism capable of synthesizing more of the compound in question must be obtained. Herein lies much of the essence of industrial microbiology. The discovery of an organism capable of a desired biochemical activity, the search for its required optimum conditions, then a search for a superior organism which is then studied to determine the optimum conditions for production.

This superior culture may belong to a quite different taxonomic category than does its predecessor, or it may belong to the same genus and species. It may be a resident of an established culture collection, as was *Penicillium notatum* NRRL 832,[20] or it may be isolated from natural sources as was *Penicillium chrysogenum* NRRL 1951.[20] Eventually, however, this superior culture will probably be a natural or induced variant of its predecessor. This latter procedure of successive mutation and strain selection of increasingly superior cultures has proved to be the most efficient method of increasing yields of microbially derived pharmaceutical products. Now let us discuss some examples of this application.

Examples of Increased Productivity Through Use of Natural and Induced Variants

The well-known story of penicillin need not be detailed. The cooperative wartime efforts of the groups at the Northern Regional Research Laboratory, the Carnegie Institution at Cold Spring Harbor, L. I., the University of Wisconsin, and the University of Minnesota are well documented.[20, 21, 29] The point to be made is that, except for the introduction of corn steep liquor as a medium ingredient[15, 16] and the discovery of precursors,[3, 11, 25] the remarkable increase from broth potencies of 1 or 2 u./ml. to 1000 u./ml. was a result of the introduction of natural and induced variants of *Penicillium notatum* and *P. chrysogenum*. Since termination of this cooperative effort, cultures superior to *P. chrysogenum* Wis. Q176 have been announced by Farrell,[11] the Merck laboratories,[13, 32] and the Wisconsin group.[1] Other groups have not published their results, but it may be surmised that they have done as well.

Another application of induced variation in relation to penicillin production has been reported. *P. chrysogenum* Wis. Q176 and its predecessors elaborate a soluble pigment or pigments, along with penicillin, that interferes with purification of the latter. By use of induced mutation Arima,[2] the Merck laboratories,[32] and the Wisconsin group[1] have obtained isolates in which this pigment synthesis has been blocked.

In much of the above work, corn steep liquor has been used as a medium constituent, and benzyl derivatives, as precursors, have been used to supplement the medium. Thus mutants producing increased yields of penicillin G have been obtained. Efforts have been made to select strains for production of other penicillins. Raper and Fennel[22] were successful in obtaining improved strains for penicillin X production. In addition, the Upjohn laboratories have reported efforts to select strains for synthesis of penicillin O.[5] Such studies involve strain selections for enzyme systems that couple the specific precursor to the remainder of the molecule. It is unfortunate that so little is known about these enzyme systems.

In retrospect, one might ask if the genus *Penicillium* would be used today for commercial penicillin production had other organisms received a comparable amount of study. It is known that other genera produce penicillin (Florey *et al.*),[12] the genus *Aspergillus* and particularly the *A. nidulans* and the *A. flavus-oryzae* groups being quite active in this respect.[12, 20, 6] Our unpub-

Strain	Method	Streptomycin productivity μ/ml
A		250
↓	UV*	
B		400
↓	NV†	
C		600
↓	UV	
D		1000
↓	UV	
E		1000–1500
↓	X rays	
F		1000–1500
↓	X rays	
G		1000–1500
↓	UV	
H		1000–1500
↓	NV	
I		1000–1500
↓	UV	
J		2000

FIGURE 1. Increased productivity of streptomycin by natural and induced mutants.

lished work indicates that the latter group contains the highest penicillin producers among the *Aspergilli*. One strain of *A. parasiticus* produced 60 u./ml. at 24° C, 40 u./ml. at 28° C, and 20 u./ml. at 37° C. The spores of this species, as well as of *A. flavus*, are apt to be multinucleate,[33] thus complicating selection procedures. We found little variability in penicillin production at 24° or 37° C. by *A. parasiticus*. Likewise, a strain of *A. flavus* proved to be quite stable.

Our work with *Streptomyces griseus* affords another example of the successful use of natural and induced variants to increase yields.[7, 8, 9, 10] These results are summarized in FIGURE 1.

Many of the pertinent aspects of this work have been discussed elsewhere[9, 10] and need not be detailed again. The point to be made is that, by selecting natural and induced variants, we have been able to obtain cultures with a productivity eightfold that of the starting culture. The yields noted in FIGURE 1 were obtained by comparing the strains in a uniform environment. This is emphasized, for each culture may require a different combination of conditions for maximum production. For example, when optimum conditions for strain J are employed, its superiority becomes more marked. Slight changes in temperature, as well as in agitation and aeration result in production of broth potencies of 2300 u./ml. by this culture.

It is quite possible that each improved variant will require a different combination of conditions for best production. It is also possible that screening variants in a set fermentation environment may result in selection of cultures that are superior within narrow limits of this environment. This possibility applies particularly to the nutrients offered the organism, for medium specific

* Ultraviolet light.
† Natural variant.

mutants must be expected. These possibilities should be considered thoroughly at the onset of selection studies.

Much has been said previously and at this conference about variability of representatives of *Streptomyces*. Further investigations into the cytology and genetics of this genus are certainly in order. The natural variability of members of the genus can be increased by treatment of spores with mutagenic agents. Thus a wide variation in single colony isolates may be obtained. One of the most variable characters is degree of sporulation, the variants ranging from colonies with no spores to those with abundant sporulation. Color of spores is another variable characteristic. Albino colonies of *S. griseus* and other species with colored spores are easily obtained, as well as mutants with spore colors that may be intermediate between albino and the pigment in the parent culture spores. Changes in the production of soluble pigment can be noted quite easily. The margin and surface of colonies that develop from spores surviving treatment with mutagenic agents also may change markedly. Some of these mutant types are shown in PLATES 1 and 2.

We have not been able to correlate any morphological type with streptomycin production, and others have likewise been unable to do so.[4, 31] Whereas Schatz and Waksman[24] report nonsporulating isolates to produce no streptomycin, we could find no such correlation. Some of our nonsporulating strains produced no streptomycin, whereas others yielded as much as the strain from which they were derived. We have reported one possible correlation between morphological type and productivity.[10] From an albino strain, a number of isolates was obtained that presented a ragged appearance, with reduced sporulation and phagelike plaques. These cultures produced streptomycin broth potencies slightly higher, but significantly so, than the albino parent or the immediate progenator of the albino, the productivity of these two being comparable. In retrospect, it is thought probable that these isolates contained phage and the higher broth potencies could be attributed to a more complete lysis of the mycelium, which would release more streptomycin into the fermentation broth. We have some preliminary results indicating that comparable increases can be obtained by breaking up growing mycelium of the correct age with ultrasonic waves.

Our superior isolates are morphologically similar to the starting culture with the exception that some may produce spores of lighter pigmentation. In addition, none of them has absolute requirements for amino acids, vitamins, purines, or pyrimidines. Only two physiological differences have been noted in any of the mutants. Some of the superior mutants produce quite high broth potencies in a synthetic medium containing l(−) proline as the sole nitrogen source. Synthesis of vitamin B_{12} also has been interrupted in the highest producing mutant.[8] In so far as can be determined, this block is complete. If the mutant is synthesizing any vitamin B_{12}, it is bound in such a way that it is not detectable by the usual assay methods.

Two other successful applications of selection of natural and induced variants to increase productivity will be noted. One of these methods concerns our work with the production of an undescribed *Streptomyces* antibiotic. Mutants were obtained that produced broth titers threefold that of the starting cultures

in a small program in which less than 2000 cultures were tested. Similar success was attained with a bacitracin-producing strain of *Bacillus subtilis*. Bacitracin broth potencies were doubled by testing a few hundred isolates.

One failure in application of this technique may be recorded. In their study of riboflavin by *Ashbya gossypii*, Pridham and Raper[19] note "unfortunately, no substrains were isolated which proved significantly superior to the parent strain in repeated comparative tests." In this study, 728 substrains were examined. Our attempt to obtain markedly superior mutants of *A. gossypii* were likewise unsuccessful. The number of isolates tested in our study was not large, but, if a comparison can be made with such studies of other cultures and products, some improved strains might have been expected. The reasons for failure to isolate superior strains is not known. Perhaps success with other cultures has been attained too easily and much larger numbers of isolates must be examined. It is difficult to calculate the possible production limits of a culture, and there is no *a priori* reason for believing the limit has been reached in this instance. It would be interesting to learn the results of other research efforts with this organism.

Speculation on Mechanisms Operating in Mutants to Produce Increased Yield

One intriguing aspect of these selection studies lies in the possible mechanism or mechanisms by which these superior variants are able to produce more of the desired compound. Until some insight is gained into the biosynthetic pathways, both directly and indirectly concerned, our thoughts in this area must remain in the realm of speculation. There are many permutations to this question. For example, increased broth titers might be obtained by selecting a variant capable of overcoming some inhibitor, present in the medium, that was blocking, even partially, some step concerned in the synthesis. The organism also may be producing such an inhibitor, this inhibition being blocked by mutation. Another strain might be selected that would undergo more complete lysis, thus liberating more efficiently a product synthesized within the mycelium. Not to be overlooked is the possibility that increased yields could be attributed to a block in degradation or transformation of the compound in question. Assays of a fermentation broth may reveal not the total amount of the product synthesized but the difference in amount synthesized and destroyed. Any decrease in rate of destruction might accordingly result in an apparent increase in the amount synthesized.

There are other facets to this problem, but one I should like to treat in more detail. No claim to originality is made here, for this subject has been discussed with others at various times. Moreover, this is the scheme to which many persons reflecting on this problem might well turn. Let us visualize the synthesis of a product *X*, as in FIGURE 2.

Starting with a common pool of nutrients required for growth and synthesis of *X*, an organism synthesizes stepwise a series of intermediates here labeled *A*, *B*, *C*, *D*, *etc.* For purposes of simplicity, two pathways leading to the moieties E and J are listed. These moieties when formed are combined to yield *X*. The fate of compound *X* is extremely important to the overall scheme. It may be degraded or further transformed, thus an equilibrium

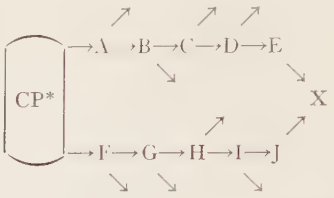

FIGURE 2. Scheme by which mutation effects increased synthesis of X.

might be established between its synthesis and further transformation. Let us consider for the moment, however, that compound X, once formed, is relatively stable and not toxic to the organism forming it. It may also be seen in FIGURE 2 that the intermediates are involved in side reactions, thus indicating that these are intermediates in other reactions. Again, for purposes of simplification, only a few alternate pathways are indicated. It is quite logical to assume that mutations operate by blocking the alternate pathways of these intermediates, thus shunting more of the intermediate into the desired reaction. Conversely, a mutation that results in blocking of the reaction $A \rightarrow B$ would stop synthesis of product X. Arrows might as well be drawn in that point toward the steps from A to B to C *etc.*, thus indicating inhibitors. These factors are omitted, however, merely for simplification. However, blocking of the undesired transformations of intermediates A, B, C, and/or D could conceivably result in synthesis of more of the moiety E. If synthesis of E was limiting synthesis of X, then more of X should be produced. If synthesis of the moiety E was not limiting formation of X, then more X would not be expected. Our data, with mutants producing increased amounts of streptomycin and penicillin, support this scheme. If the above scheme is operating, one might expect that productivity would go up stepwise with each mutant in which a block in an alternate pathway occurred. This is apparently what happens, because yields have increased stepwise with each improved culture. Likewise, one would expect increased synthesis of X when increased levels of nutrients are fed to the improved mutant. This increase also frequently occurs. When a series of mutants is obtained, each series successively capable of improved synthesis, it might be expected that many of the alternate pathways of the intermediates have been blocked. Improved mutants should accordingly be increasingly difficult to obtain. Our data support this hypothesis.

Our results, however, contain one disturbing point with respect to this scheme. For example, if one has a series of mutants capable of producing 100, 500, 1000, 2000 and 4000 μg. ml. of product X, it should be as easy to interrupt the synthesis of X in the mutant synthesizing 4000 μg./ml. as in the mutants elaborating much less of the product. We are assuming here that the same synthetic pathways are operating in the series of mutants; the same genetic loci controlling these synthetic steps are therefore available for induced mutations. If the spores of this series of improved mutants are accordingly treated with a mutagenic agent and the isolates derived from the surviving spores are tested for productivity, one should expect, within limits, as many nonproducers

* CP = common pool of nutrients.

from the best strains as from the inferior strains. The lowest producers in this mutant series might be an exception. For any stepwise decrease here might result in yield of product X too low to measure.

Results we have obtained with mutants of *Streptomyces griseus* and *Penicillium chrysogenum* do not support this hypothesis. Nonproducing mutants are increasingly difficult to obtain from strains producing high broth potencies of streptomycin and penicillin. The highest producing cultures yield no nonproducers. There is, however, no indication of a corresponding decrease in phenotypic mutants. Perhaps our series of mutant strains is unusual. If so, it might be possible to explain away this contradiction. If this condition prevails in the mutant series of other laboratories, however, it presents a formidable obstacle to the scheme outlined in FIGURE 2.

The scheme deserves comment on two other points. It is theoretically possible to increase one of the pathways without increasing the yield of X if this is being limited by synthesis of the other moiety. Accordingly, if one attempts to increase the yield of penicillin, streptomycin, or another product without success, it might be advisable to use other cultures for mutation studies. Logically, isolates of the culture, used without success, might well be studied even if these isolates did not produce more of the product. The rationale is that this isolate might produce more of one limiting moiety of the desired molecule, and increased yield of another limiting moiety might be obtained more readily. It might even be expedient to return for further mutation study to inferior predecessors of the culture to be abandoned, although this step does not necessarily follow from the above arguments. The other point that deserves comment is intimately related to the recombinant technique of Pontecorvo and his associates and will be discussed below.

Significance of Recombination in Imperfect Fungi

The use of natural and induced variability has been quite successful. However, the range of such studies is limited. The scope of what may be accomplished with the imperfect fungi, however, has now been greatly expanded by the studies of Pontecorvo and Roper (reviewed in detail[15]). The implications of this work should not be lost to the industrial field, and students of taxonomy and phylogeny should continually be aware of its importance. Through the use of nutritionally deficient mutants, Pontecorvo and his associates have been able to effect the formation of heterocaryons. From the latter, heterozygous diploids have been obtained. These heterozygous diploids undergo segregation with the resultant formation of diploid and even haploid recombinants. This technique may accordingly be used to obtain recombinant strains carrying desired characters found in different strains. A few possibilities may be cited. Vitamin B_{12} synthesis has been blocked in one of our superior streptomycin-producing mutants of *S. griseus*. It would therefore be expedient to obtain a recombinant of this mutant and another vitamin B_{12} producing strain that is capable of producing high yields of both streptomycin and vitamin B_{12}. Another example is illustrated by the point in the preceding paragraph that was referred here for discussion. A mutant might be obtained that produced excessive amounts of one moiety of a desired molecule. An-

other mutant might produce large amounts of another moiety. Neither might elaborate more of the desired product. Recombinants of these and other mutants that synthesize excessive amounts of complementary portions of the desired compound, however, could well increase the over-all yield of the product. Another apt illustration is the use of this recombinant technique in the study of mutants in which synthesis of a product has been blocked. Recombinants of such mutants might aid in studying biosynthetic mechanisms. It might have been expected that use of heterocaryons would have solved some of these problems. The behavior of heterocaryons, however, is unpredictable.

As a final point, the significance of ploidy in synthesis of a product might now be examined. It should be possible to obtain a series of polyploids and study the relationship of increased chromosome complement and synthesis of a product.

It is not inferred that the above examples await an easy solution. Tools for this work, however, now seem to be at hand.

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PLATE 1. Ultraviolet light induced mutants of *Streptomyces griseus* growing on yeast extract-glucose agar. Approximately 4×.

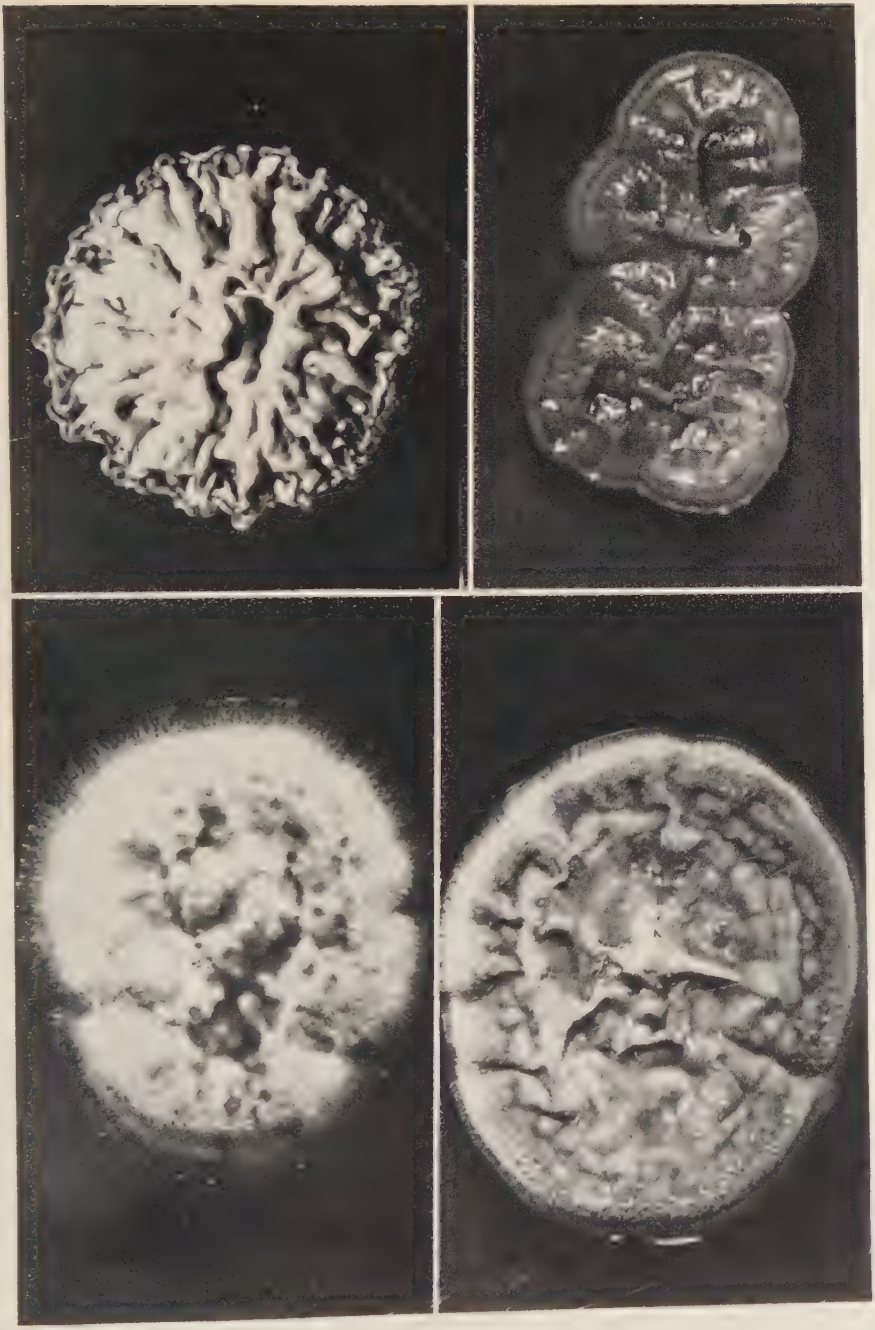
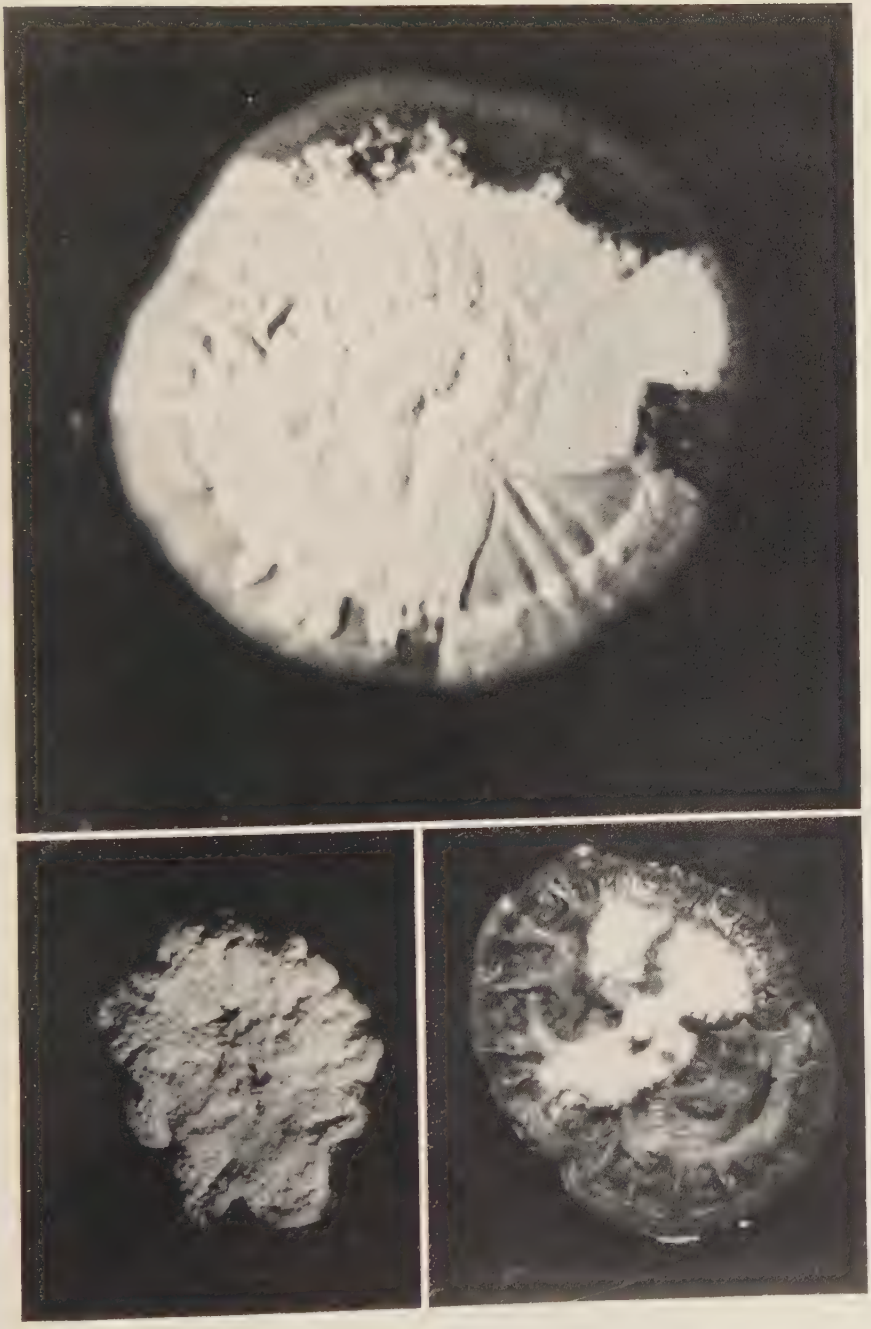


PLATE 2. Ultraviolet light induced mutants of *Streptomyces griseus*. Lower two colonies are growing on yeast extract-glucose agar. Approximately 4×. Upper colony illustrates sectoring that may be encountered in some strains. This colony had developed for two weeks on a medium composed of d-xylose, N-Z-Amine and inorganic salts.



MORPHOLOGICAL STUDIES IN THE GENUS *NOCARDIA*. III. THE MORPHOLOGY OF YOUNG COLONIES*

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The genus *Nocardia* was proposed by Waksman and Henrici (1943) to include obligatively aerobic actinomycetes that produce branched mycelia which are transitory and sooner or later fragment into "oidiospores or segmentation spores." The genus is placed in the family *Actinomycetaceae* by these authors along with the anaerobic genus *Actinomyces*. Actinomycetes which form permanent mycelia that do not fragment were placed in the family *Streptomycetaceae*. Organisms belonging to this family also bear conidia in aerial hyphae. The genus *Nocardia* includes species which regularly produce aerial hyphae, but conidia are not cut off in these species. The basis for separation of the genus *Nocardia* from the genus *Streptomyces* is the permanence of the mycelium in the latter genus. The boundaries of the genera are thus laid on a morphological basis.

It is often difficult to decide whether or not a particular microorganism produces a mycelium, a term that has not been adequately defined as was pointed out by Umbreit (1939). In 1949 the author suggested, without much enthusiasm, that a workable definition might be a "branched hyphae at least 10 microns long." If one accepts this empirical concept, he can usually differentiate between branched bacteria (*Mycobacterium* and *Corynebacterium*) and an actinomycete. However, there are strains in which the mycelium is very ephemeral and observations must be made on very young cultures in order to recognize its presence. On the other hand, there are nocardias which form a mycelium that is quite permanent, fragmentation occurring only in old cultures. Between these two extremes one can find strains which exhibit all intermediate degrees of mycelial formation.

Since the principal criterion for the genus *Nocardia* is based on the degree of permanence of the mycelium, the author carried out studies in 1949 in an effort to determine to what degree the mycelium is permanent in different species. By studying single cells as they grew into young colonies, it was found that the genus can be divided into three morphological groups on the basis of the permanence of mycelium. Other morphological characteristics, such as time of branching, method of fragmentation, pigment production, occurrence of substrate and aerial hyphae, were correlated with the morphological groups. For convenience, the characteristics of these three groups are summarized here:

GROUP I. Characterized by sparse branching, early fragmentation (often within 12 hours) and fragmentation frequently occurring at the bends in hyphae, so that the daughter cells come to lie parallel to each other, similar to the "snapping" movements described for certain bacteria. This fragmentation was designated as of TYPE 1.

GROUP II. Characterized by the production of a well-defined mycelium

* This study was supported in part by a grant from the General Research Fund of the University of Kansas.

which is richly branched. Fragmentation occurs later than in GROUP I, and usually occurs in such a way that the fragmented ends continue to grow in their original directions (designated as fragmentation of TYPE 2), or a multiple fragmentation at the base of a branch (TYPE 3 fragmentation). Fragmentation of TYPE 1 is lacking or infrequently present in organisms belonging to this group. The ends of branches of organisms in this group grow parallel to each other when they touch and do not usually cross.

GROUP III. Characterized by a diffuse, much branched mycelium which fragments only after several days' growth. The branches of organisms belonging to this group are often contorted. They also overlap and cross each other freely. Substrate and aerial hyphae are frequently produced. The colonies are characteristically wide spread, and branches are placed farther apart than in organisms of either GROUPS I or III. Organisms in this group may produce soluble as well as intracellular pigments. The pigments of organisms in GROUPS I and II are always intracellular.

From these studies it was concluded that organisms which would fall within the limits of the genus *Nocardia* differ considerably in morphology. Strains were studied which exhibited a somewhat intermediate behavior between these three groups, and the question as to whether they belonged to the one group or the other required careful study. Organisms in the genus *Nocardia*, then, form a morphological series ranging from those that are quite like bacteria to those that are very like *Streptomyces*. Unless one accepts the presence of conidia in aerial hyphae as an additional criterion for separating *Nocardia* species from *Streptomyces* species, there are many actinomycetes that on the basis of fragmentation alone would be *Streptomyces* species rather than *Nocardia* species. The question of asporogenous *Streptomyces* species has not been adequately studied.

In order to study the developmental morphology as a possible criterion for speciation in the genus *Nocardia*, the following studies were made. Thirty-seven strains of *Nocardia* species were grown on the surface of agar blocks and observations were made of them at about 12-hour intervals. In order to eliminate as much as possible the influence of the medium on the morphology a semisynthetic medium having the following composition was used: glucose, 20.0 g.; $NaNO_3$, 2.0 g.; K_2HPO_4 (anhydrous), 1.0 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $Fe_2(SO_4)_3$, 10.0 mg.; $MnCl_2 \cdot 4H_2O$, 8.0 mg.; $ZnSO_4 \cdot H_2O$, 2.0 mg.; agar 20.0 g., distilled water to make 1000 ml. pH adjusted to 7.0 to 7.2. The technique was the same as that previously described (1949). Between observation times, the cultures were incubated at 28° C. The inoculum used was always from slants that were at least two months old, so that the ultimate products of the growth of the organism could be used. Inoculum was made by suspending cells in 2 ml. of sterile distilled water. Mycelium of strains which did not suspend readily was ground against the sides of the tube with a sterile glass rod, to make a uniform suspension. A small loopful of this suspension was then streaked onto the agar blocks. The cultures were prepared in duplicate and placed in moist chambers for incubation. Observations were made after 12, 24, 36, 48, and finally 60 to 72 hours. At the time of observation, notes were made as to the degree of mycelial de-

velopment, average number of branches, whether or not fragmentation had occurred, type of fragmentation, and general morphology of the developing colony. After looking over the slides, one or a group of specimens was selected for photographing. In all cases there was considerable difference in the development of individuals. Usually there were some cells that never germinated. If cells were close together or in clumps they germinated faster than separate cells. The hyphae or young colonies that were selected for photographing were those not too close to other cells, and those which were repre-

TABLE 1
LIST OF ISOLATES STUDIED

Stock No.	Name	Source	Morphological group
1	*Proactinomyces ruber* (Casabó) Bald.[1]	Centraalbureau voor Schimmelcultures, Baarn	2
2	*Nocardia* sp.	Soil	2
4	*P. agnosus*	CBS	1
6	*N. polychromogenus* (Vallée) Bergey *et al.*	CBS	1
8	*N. erythropolis* (Gray and Thornton) Waks.	Waksman 3407	1
9	*P. restrictus* Turfitt	CBS	1
10	*N. asteroides* var. *crateriformis* (Bald.) Bergey *et al.*	CBS	2
12	*Nocardia* sp.	Oil sands	2
14	*Nocardia* sp.	K. L. Jones	1
15	*Nocardia* sp.	Soil	1
16	*Nocardia* sp.	Soil	1
33	*Nocardia* sp.	Soil	2
42	*Nocardia* sp.	K. L. Jones	2
52	*Nocardia* sp.	O. A. Plunkett	3
57	*Jensenia canicruria* Bisset and Moore	F. A. Clark	1
74	*N. rubra* (Krassilnikov) Bergey *et al.*	NRRL B-685	2
75	*N. globerula* (Gray) Bergey *et al.*	NRRL B-1306	2
76	*N. opaca* (den Dooren de Jong) Bergey *et al.*	American Type Culture Collection 4276	2
77	*N. rangoonensis* (Erikson) Bergey *et al.*	ATCC 6860	3
78	*N. corallina* (Bergey *et al.*) Bergey *et al.*	ATCC 4273	2
79	*N. convoluta* (Gray and Thornton)[2]	ATCC 4275	2
80	*N. cuniculi* Snijders	ATCC 6864	3
83	*N. sylvodorifera* A. Cast.	ATCC 7372	3
84	*N. asteroides* (Eppinger) Blanchard	ATCC 3308	3
87	*N. polychromogenes* (Vallée) Bergey *et al.*	CBS	2
90	*P. aquosus* Turfitt	CBS	1
91	*N. caviae* Snijders.	CBS	2
92	*N. asteroides* (Eppinger) Blanchard	Emmons 9903	3
93	*N. asteroides* (Eppinger) Blanchard	Emmons 9935	3
94	*N. asteroides* (Eppinger) Blanchard	Emmons 9976	3
95	*N. asteroides* (Eppinger) Blanchard	Emmons 9977	3
97	*N. asteroides* (Eppinger) Blanchard	Emmons 9974	3
101	*N. asteroides* (Eppinger) Blanchard	Emmons 9955	2
103	*N. minima* (Jensen) Bergey *et al.*	ATCC 8674	3
104	*N. madurae* (Vincent) Blanchard	ATCC 6245	3
106	*N. gardneri* (Waksman) Bergey *et al.*	ATCC 9604	3
111	*N. asteroides* (Eppinger) Blanchard	Pasteur Institute 502	2

[1] The old genus name *Proactinomyces* is retained here for cultures that have not been listed as synonyms for valid species in the Sixth Edition of Bergey's Manual for Determinative Bacteriology.
[2] This organism is the *Mycobacterium convolutum* of Gray and Thornton.

sentative of the "average" as nearly as could be judged. No attempt was made to select a special cell for study, as a general picture of the developmental morphology was desired. The cultures studied and their origins are shown in TABLE 1.

A brief description follows of the morphology of the cultures listed in TABLE 1, at the time intervals stated:

Morphological Group I

STOCK No. 4. *Proactinomyces agnosus*. FIGURES 1, 2, 3, and 4.
12 hrs. Rods elongating, primary branching beginning.
24 hrs. Branched rods have produced small colonies by fragmentation of types 1 and 2.
36 hrs. Small colonies produced by multiple fragmentation of filaments. Short, curved hyphae remain at the edges of colonies.
72 hrs. Small colonies composed of short fragments with short hyphae at periphery produced.

STOCK No. 6. *Nocardia polychromogenes* (Jensen) Waksman. FIGURES 129, 130, 131 and 132.
12 hrs. Short rods, no germination.
15 hrs. Rods beginning germination by elongation.
24 hrs. Rods bend forming C- and S-shaped hyphae, small branches produced.
36 hrs. Bent and curved hyphae continue to elongate, branches sparse and short.
72 hrs. Small colonies, central hyphae fragmented, peripheral hyphae continue to grow and branch.

STOCK No. 8. *N. erythropolis* (Gray and Thornton) Waksman. FIGURES 5, 6, 7, and 8.
12 hrs. Rods elongating and bending.
15 hrs. Bent rods begin to fragment (type 2), few short branches produced.
24 hrs. Small colonies composed of few long, fragmenting hyphae.
36 hrs. Small colonies consist of fragmented short cells in center. Peripheral hyphae grow, bend and fragment.
72 hrs. Large circular colonies, center dense, hyphae at edges bent, but sparsely branched.

STOCK No 9. *P. restrictus* Turfitt. FIGURES 9, 10, 11, and 12.
12 hrs. Rods germinate by elongation.
24 hrs. Small colonies assume the shape of the branched filament. Fragmentation multiple along hyphae.
36 hrs. Small colonies tend to become circular as fragmentation proceeds. Hyphae are very short, those on edges continue to grow, bend and fragment.
62 hrs. Fragmentation of filaments general, colonies with almost entire margins produced.

STOCK No. 14. *Nocardia* sp. FIGURES 13, 14, 15 and 16.
12 hrs. Short rods elongating and bending.
24 hrs. Young colonies composed of long fragmented hyphae. Few branches produced. Fragmentation of types 1 and 2 begins in center of small colony.
36 hrs. Large colonies consisting of long hyphae with few branches.
72 hrs. Large dense colonies which fragments in center, peripheral hyphae continue to grow, branch and fragment. Fragmentation frequently of type 1.

STOCK No. 15. *Nocardia* sp. FIGURES 21, 22, 23, and 24.
12 hrs. Refractive rods germinating by elongation.
24 hrs. Young colonies formed by fragmentation into long hyphae. Few branches and fragmentation is of types 1 and 2.
36 hrs. Colonies formed by long unbranched hyphae continue to elongate at the edges. Fragmentation produces shorter cells.
70 hrs. Circular colonies, dense in center, radiating, nonbranched hyphae around periphery.

STOCK No. 16. *Nocardia* sp. FIGURES 17, 18, 19 and 20.
12 hrs. Bent rods, some forming short branches.
24 hrs. Small colonies consisting of fragmented hyphae. Few short branches produced.
36 hrs. Circular colonies with short fragments in the center. Hyphae mostly unbranched around periphery.
70 hrs. Large circular dense colonies, short bent peripheral hyphae.

STOCK No. 57. *Jensenia canicruria* Bisset and Moore. FIGURES 25, 26, 27 and 28.
12 hrs. Rods elongating and bending, few short branches produced.
24 hrs. Small colonies consisting of sharply bent hyphae in center, with longer curved hyphae around edges. Branches few. Fragmentation of type 1

FIGURES 1 through 32 are *Nocardia* species belonging to Morphological Group I. The four columns represent the appearance of the organisms at 12, 24, 36, and 72 hours respectively. FIGURES 1–4 *Proactinomyces agnosus*; FIGURES 5–8 *Nocardia erythropolis*; FIGURES 9–12 *P. restrictus* FIGURES 13–16 Unidentified *Nocardia* species from soil, Stock No. 14. FIGURES 17–20 Unidentified *Nocardia* species from soil; Stock No. 16, FIGURES 21–24 Unidentified *Nocardia* species from soil; Stock No. 15. FIGURES 25–28 *Jensenia canicruria*; FIGURES 29–32 *P. aquosus*.

36 hrs. Circular colonies consisting of short hyphae in center with longer curved hyphae at periphery.
70 hrs. Larger circular colonies dense in center with curved nonbranched hyphae on edges.

Stock No. 90. *P. aquatus* Turfitt. Figures 29, 30, 31, and 32.
12 hrs. Rods elongating, bending, branching and fragmentation of type 1. First branch frequently in the air.
24 hrs. Small colonies fragmented in center, few hyphae at edges.
36 hrs. Fragmentation of type results in small colonies the fragments of which produce a zig-zag arrangement.
70 hrs. Small circular colonies, amorphous in shape, almost completely fragmented with margins having only a few hyphae evident.

Morphological Group II

Stock No. 1. *Proactinomyces ruber* (Casabó) Bald. Figures 33, 34, 35, and 36.
12 hrs. Rods elongating, with few short branches produced.
15 hrs. Rods continue to elongate bend and branch.
24 hrs. Secondary branches produced on richly branched mycelium.
36 hrs. Extensively branched mycelium, fragmentation of types 2 and 3 beginning in center.
72 hrs. Large colonies with fragmentation into long hyphae in center, long branched hyphae at periphery.

Stock No. 2. *Nocardia* sp. Figures 125, 126, 127, and 128.
12 hrs. Pleomorphic rods beginning to germinate by elongation.
24 hrs. Hyphae branching, fragmentation of type 2 beginning in center.
36 hrs. Central hyphae continue to fragment, hyphae at edges continue to grow and branch.
72 hrs. Dense circular colonies produced, hyphae in center very fragmented, branched hyphae at edges.

Stock No. 10. *N. asteroides* var. *cerebriformis* (Bald.) Bergey *et al.* Figures 133, 134, 135, and 136.
12 hrs. Short rods and cocci, no germination.
24 hrs. Elongating rods.
36 hrs. Elongating branched rods. Few small colonies are produced.
62 hrs. Small colonies produced that are sparsely branched. Branches are long, with few branches, and short aerial hyphae are produced. No fragmentation.

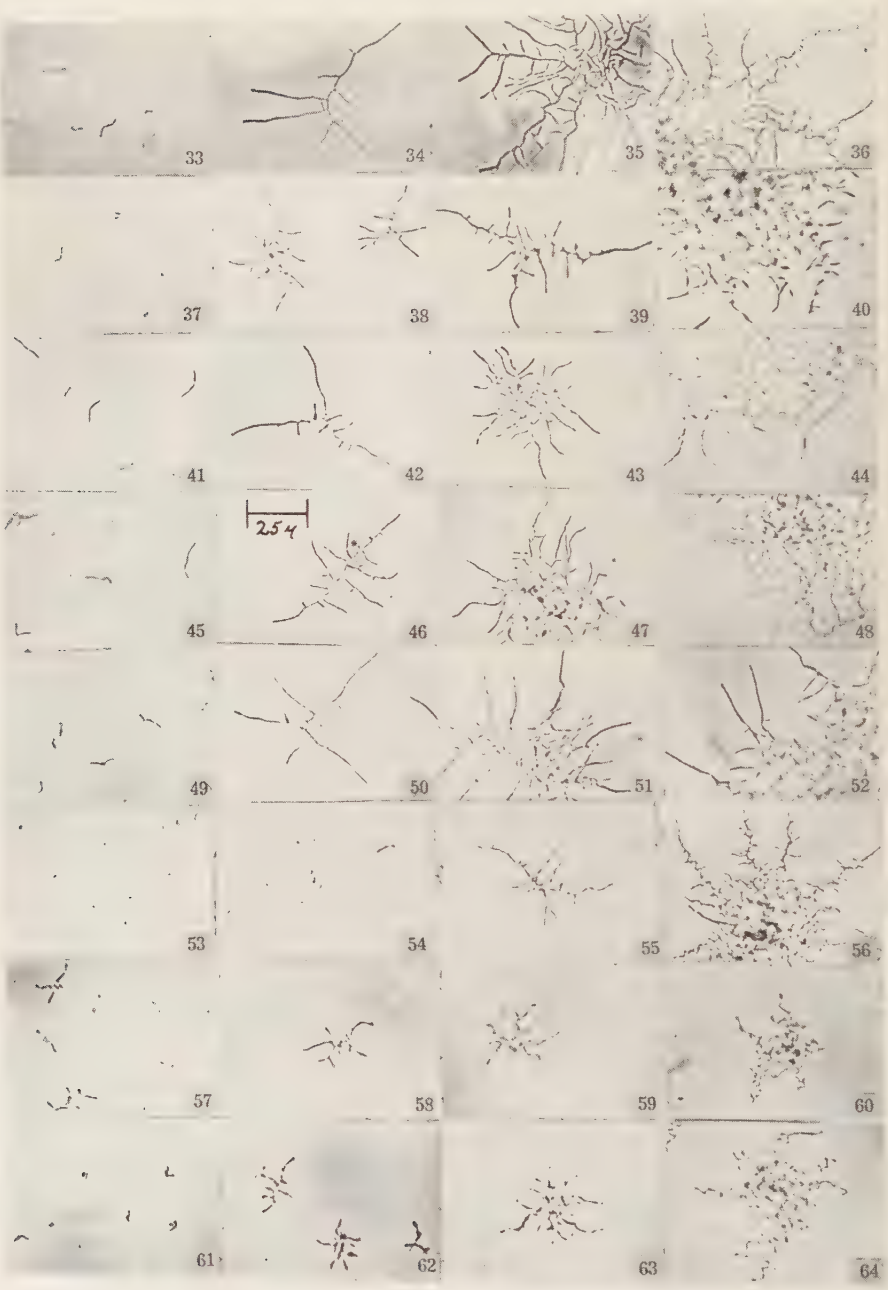
Stock No. 12. *Nocardia* sp. Figures 37, 38, 39, and 40.
12 hrs. Rods germinating by elongation. Hyphae curving.
24 hrs. Colonies produced by hyphae elongating and branching. Secondary branching has occurred. Type 2 fragmentation beginning in the center of colony.
36 hrs. Large branched colonies, fragmentation continues in center.
62 hrs. Large circular colonies consisting of fragments in center, with long branched hyphae at periphery.

Stock No. 33. *Nocardia* sp. Figures 41, 42, 43, and 44.
12 hrs. Rods germinating by elongation. Hyphae curving.
24 hrs. Long branched filaments forming young colonies. Type 2 fragmentation beginning in the center.
36 hrs. Circular colonies, peripheral hyphae long with few branches. Fragmentation continues in center.
62 hrs. Large circular colonies, very fragmented in center, long branched hyphae at periphery.

Stock No. 42. *Nocardia* sp. Figures 45, 46, 47, and 48.
12 hrs. Rods germinating by elongation. Short primary branches formed.
24 hrs. Large branched colonies with secondary branching beginning. Type 2 fragmentation beginning in center of colony.
36 hrs. Fragmentation continues in center, peripheral hyphae long and branched.
70 hrs. Very large dense colonies produced. Hyphae at edge of colony continues to grow, branch and fragment at bases.

Stock No. 74. *N. ruber* (Krassilnikov) Bergey *et al.* Figures 49, 50, 51, and 52.
12 hrs. Elongating rods with few short branches.
24 hrs. Large young branched colonies. Fragmentation of type 2 beginning in center.
36 hrs. Large colonies with fragmentation in center, peripheral branches continue to elongate and branch.
76 hrs. Large colonies, fragmented in center with long branched peripheral hyphae.

Stock No. 75. *N. globerula* (Gray) Bergey *et al.* Figures 137, 138, 139, and 140.
12 hrs. Cocci and rods, no germination.
24 hrs. Rods germinating by elongation. Hyphae bending.



FIGURES 33 through 64 are *Nocardia* species belonging to Morphological Group II. The four columns represent the appearance of the organisms at 12, 24, 36 and 72 hours respectively. FIGURES 33 36 *P. ruber*; FIGURES 37–40 Unidentified *Nocardia* species from oil sand; Stock No. 12; FIGURES 41–44 Unidentified *Nocardia* species from soil; Stock No. 33, FIGURES 45–48 Unidentified *Nocardia* species from soil; Stock No. 42, FIGURES 49–52 *N. rubra*; FIGURES 53–56 *N. caviae*; FIGURES 57–60 *N. opaca*; FIGURES 61–64 *N. corallina*.

36 hrs. Small colonies with fragmentation in center, few branches produced.
72 hrs. Small compact colonies with fragmented center and short peripheral hyphae.

Stock No. 76. *N. opaca* (den Dooren de Jong) Bergey *et al.* Figures 57, 58, 59, and 60.
12 hrs. Rods elongating, bending, and producing short branches.
24 hrs. Small colonies with secondary branching, type 2 fragmentation beginning in center.
36 hrs. Small colonies with hyphae at periphery with few branches, fragmentation in center.
72 hrs. Small compact colonies with short fragments in the center, short peripheral hyphae produced.

Stock No. 78. *N. corallina* (Bergey et al.) Bergey *et al.* Figures 61, 62, 63, and 64.
12 hrs. Short elongating rods, some bending.
24 hrs. Branched hyphae forming small colonies. Fragmentation of type 2 beginning in center.
36 hrs. Colonies consist of fragmented central hyphae, peripheral hyphae sparsely branched.
72 hrs. Central hyphae fragmented to short rods, arranged at angles. Peripheral hyphae characteristically branched.

Stock No. 79. *N. convoluta* (Gray and Thornton). Figures 141, 142, 143, and 144
12 hrs. Curved elongating rods, occasional short branches produced.
24 hrs. Rods elongating and branching. Branches are produced so as to suggest dichotomous branching. Fragmentation occurring in center.
36 hrs. Circular colonies with curved branching hyphae at periphery.
70 hrs. Large colonies with dense center, long branched hyphae at periphery.

Stock No. 87. *N. polychromogenes* (Vallée) Bergey *et al.* Figures 145, 146, 147, and 148.
12 hrs. Short rods not germinating.
24 hrs. Rods elongating with short branches.
36 hrs. Rods forming small branched colonies which are fragmenting.
72 hrs. Colonies with peripheral hyphae having few branches. Fragmentation produces long hyphae in center.

Stock No. 91. *N. caviae* (Snijders). Figures 53, 54, 55, and 56.
12 hrs. Short non-germinating rods.
24 hrs. Rods germinating by elongation.
36 hrs. Branched colonies with fragmentation of types 1 and 2 beginning in center. Short aerial hyphae produced.
70 hrs. Colonies with fragmentation in center. Peripheral hyphae profusely branched and fragmented. Short aerial hyphae in center of colony.

Stock No. 101. *N. asteroides* (Eppinger) Blanchard. Figures 117, 118, 119, and 120.
12 hrs. Rods and cocci, no germination
24 hrs. Rods elongating, and branching.
36 hrs. Rods elongating and branching. Fragmentation in the original hyphae.
72 hrs. Colonies produced with fragmentation in center. Branched hyphae at periphery.

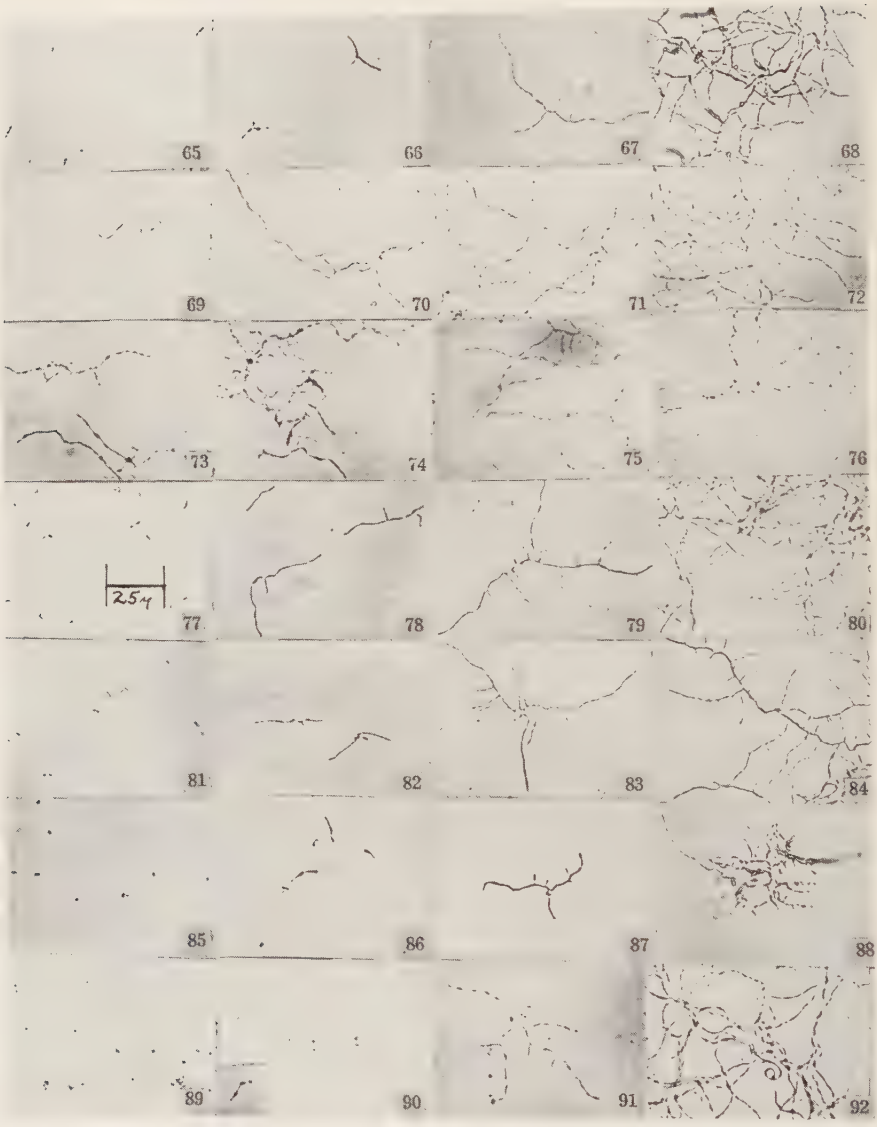
Stock No. 111. *N. asteroides* (Eppinger Blanchard. Figures 121, 122, 123, and 124.
12 hrs. germinating beginning with rods elongating and bending.
24 hrs. Elongation continues, short branches produced.
36 hrs. Short hyphae with branches produced.
60 hrs. Small colonies with branches.
72 hrs. Branched colonies with fragmentation in center. Radial hyphae with short branches.

Morphological Group III

Stock No. 52. *Nocardia* sp. Figures 85, 86, 87, and 88.
12 hrs. Rods and cocci present. No germination.
24 hrs. Rods elongating, producing a few short branches.
36 hrs. Small colonies with branches, fragmentation of type 2 in center, and aerial hyphae.
70 hrs. Branched colonies, with limited fragmentation in center. Substrate and aerial hyphae profusely produced.

Stock No. 77. *N. rangoonensis* (Erikson) Bergey *et al.* Figures 89, 90, 91, and 92.
12 hrs. Ungerminated cocci.
24 hrs. Most cells ungerminated. Few elongated curved rods.
36 hrs. Colonies consisting of hyphae with wide spread branches.
72 hrs. Long hyphae forming very diffuse, much branched mycelium. Both aerial and substrate hyphae produced with many spiraled filaments.

Stock No. 80. *N. cuniculi* Snijders. Figures 81, 82, 83, and 84.
12 hrs. Rods beginning germination by elongation.
24 hrs. Hyphae beginning to branch. Fragmentation of type 2 occasionally present.



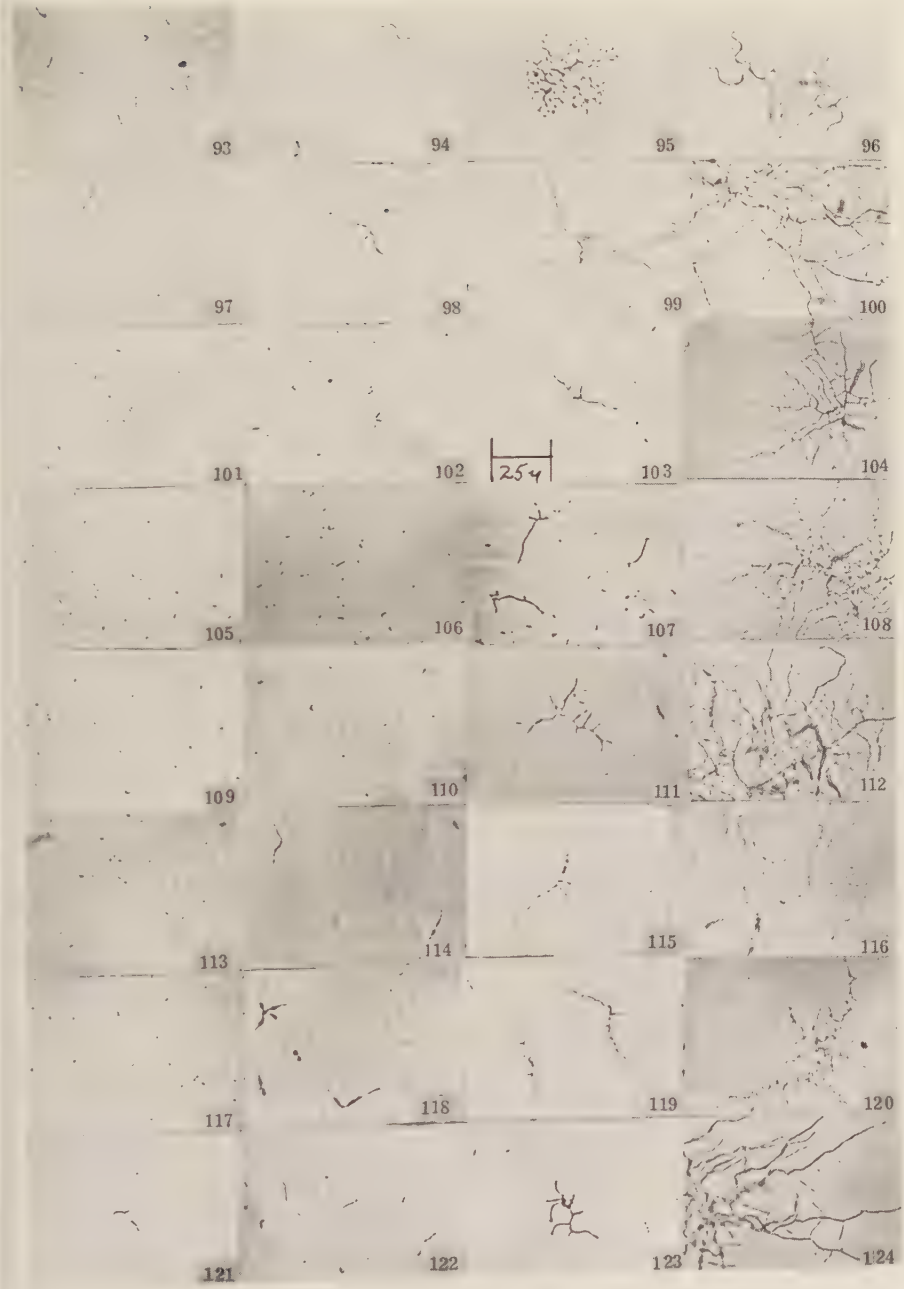
FIGURES 65 through 92 are *Nocardia* species belonging to Morphological Group III. FIGURES 65–68 *N. minima* at 12, 24, 36 and 72 hrs; FIGURES 69–72 *N. gardneri* at 12, 24, 36 and 70 hrs.; FIGURES 73–76 *N. madurae* at 24, 36, 48 and 60 hrs.; FIGURES 77–80 *N. sylvodorifera* at 12, 24, 36 and 70 hrs.; FIGURES 81–84 *N. cuniculi* at 12, 23, 35 and 72 hrs ; FIGURES 85–88 Unidentified *Nocardia* species from skin lesions; Stock No. 52, at 12, 24, 36 and 70 hrs.; FIGURES 89–92 *N. rangoonensis* at 12, 23, 34, and 70 hrs.

36 hrs. Long branched hyphae forming colony. Fragmentation is not very frequent. Aerial and substrate hyphae produced.

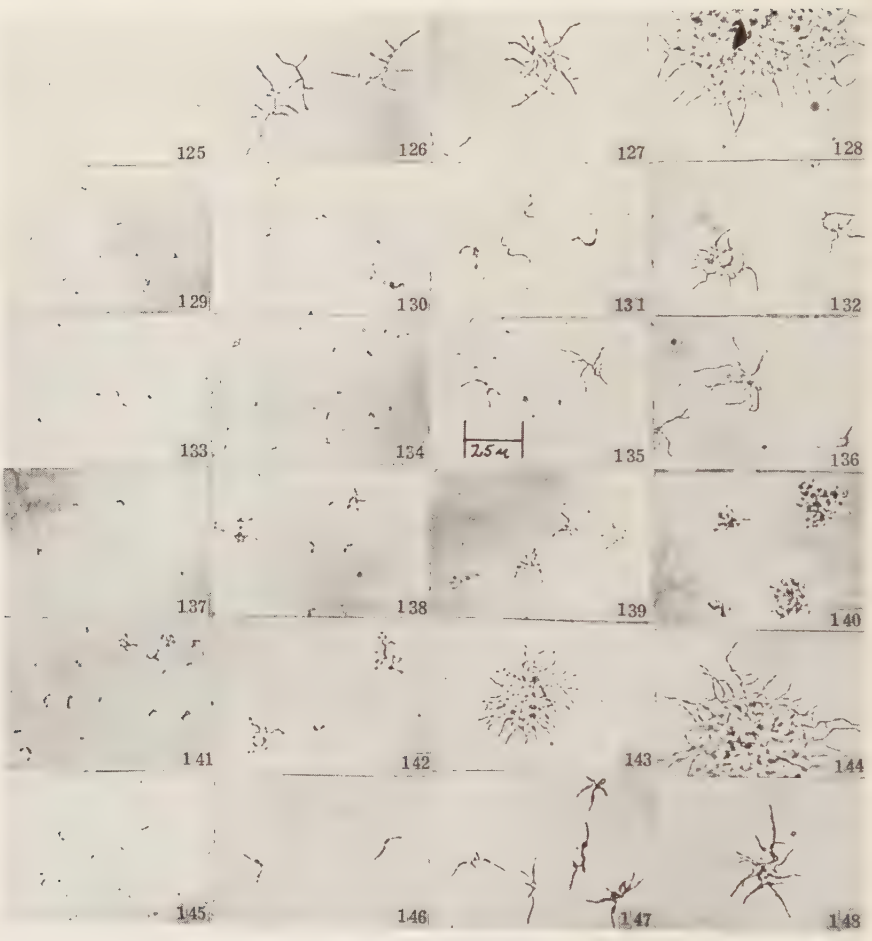
70 hrs. Colonies consisting of radiating hyphae, short aerial hyphae with slight fragmentation in center.

STOCK No. 83. *N. sylvodorifera* A. Cast. FIGURES 77, 78, 79, and 80.

12 hrs. Rods germinate by elongation.



FIGURES 93 through 124 are all isolates of *Nocardia asteroides* at 12, 24, 36 and 72 hrs. respectively. Stock Numbers reading down are 84, 92, 93, 94, 95, 97, 101 and 111.



FIGURES 125 through 148 are miscellaneous *Nocardia* species at 12, 24, 36 and 72 hrs. respectively. FIGURES 125–128 Unidentified *Nocardia* species from soil stock No. 2.; FIGURES 129–132 *N. polychromogenes*; Stock No. 6, FIGURES 133–136 *N. asteroides* var. *crateriformis*; FIGURES 137–140 *N. globerula*, FIGURES 141–144 *N. convoluta*. FIGURES 145–148 *N. polychromogenes*.

24 hrs. Long filaments beginning to branch.
36 hrs. Long branched hyphae form a diffuse colony. Aerial hyphae produced.
72 hrs. Radiating branched colonies with short aerial hyphae. Colonies very diffuse and spreading.

STOCK No. 84. *N. asteroides* (Eppinger) Blanchard. FIGURES 93, 94, 95, and 96.
12 hrs. Rods germinating by elongation.
24 hrs. Curved hyphae, S- and C-shaped, short branches beginning.
36 hrs. Curved hyphae made up the small colonies. Aerial hyphae produced. The central hyphae beginning to fragment.
72 hrs. Mycelium with characteristically curved hyphae at periphery of colonies. Slight fragmentation in center of small colonies with aerial hyphae.

STOCK No. 92. *N. asteroides* (Eppinger) Blanchard. FIGURES 97, 98, 99, and 100.
12 hrs. Rods germinating by elongation.
24 hrs. Rods beginning to branch, some of which are aerial.
36 hrs. Larger colonies consisting of diffuse branches with aerial hyphae.

70 hrs. Very diffuse colonies with many long branches which cross each other freely. Many aerial hyphae produced.

Stock No. 93. *N. asteroides* (Eppinger) Blanchard. Figures 101, 102, 103, and 104.

12 hrs. Rods and cocci, no germination.
24 hrs. Rods germinating by elongation, with an occasional short branch.
36 hrs. Small colonies with many branches, fragmentation occasionally occurs in center of colony.
72 hrs. Large diffusely branched colonies with long branched aerial hyphae. The long, widely spaced branches cross freely.

Stock No. 94. *N. asteroides* (Eppinger) Blanchard. Figures 105, 106, 107, and 108.

12 hrs. Rods and cocci, no germination.
24 hrs. Rods germinating by elongation.
36 hrs. Elongated rods with few branches.
48 hrs. Rods growing and branching is more frequent.
72 hrs. Branched colonies with aerial hyphae. Many small branches which produce a compact colony.

Stock No. 95. *N. asteroides* (Eppinger) Blanchard. Figures 109, 110, 111, and 112.

12 hrs. Rods and cocci, no germination.
24 hrs. Rods elongating slightly, beginning germination.
36 hrs. Elongating hyphae with short branches.
48 hrs. Diffuse, much branched hyphae produced.
72 hrs. Diffusely branched colonies with aerial and substrate hyphae. The branches curve and cross freely.

Stock No. 97. *N. asteroides* (Eppinger) Blanchard. Figures 113, 114, 115, and 116.

12 hrs. Rods and cocci, no germination.
24 hrs. Most cells ungerminated, a few have elongated, and begun to bend.
36 hrs. Branched colonies formed, no fragmentation evident.
72 hrs. Very diffuse branched colonies with aerial hyphae.

Stock No. 103. *Nocardia minima* Jensen Bergey *et al.* Figures 65, 66, 67, and 68.

12 hrs. Rods, elongating, no branching.
24 hrs. Elongation continues, short branches formed.
36 hrs. Colonies with few long branches produced.
60 hrs. Hyphae continue to grow and branch, forming a diffuse wide spread colony with short aerial hyphae.

Stock No. 104. *N. madurae* Vincent, Blanchard. Figures 73, 74, 75, and 76.

12 hrs. Ungerminated refractive cocci. Hyphal fragments growing.
24 hrs. Hyphal fragments continue to elongate and produce widely spaced long branches.
36 hrs. Diffuse colonies with long contorted branches. Substrate mycelium richly produced.
60 hrs. Large diffuse colonies with no fragmentation. Substrate and aerial hyphae richly produced.

Stock No. 106. *N. gardneri* (Waksman) Bergey *et al.* Figures 69, 70, 71, and 72.

12 hrs. Coccoid cells and hyphal fragments present. Fragments elongating.
15 hrs. Filaments growing that begin to branch.
24 hrs. Colonies with contorted, looping, wide spread branches.
36 hrs. Long curved filaments, with substrate mycelium. Widely spaced branches with aerial hyphae. The hyphae loop and cross freely.
70 hrs. Diffuse, wide spread colonies. Aerial and substrate mycelium richly produced.

Discussion

The results of these studies reemphasize the wide diversity that exists in the mycelial formation of organisms comprising the genus *Nocardia*. Organisms in morphological group I produce a limited amount of mycelium which varies considerably in its degree of permanency. In certain strains fragmentation begins soon after germination and sometimes before branches are evident. However, branches are always produced. Young colonies of these organisms are composed of fragmented hyphae which now and then form short branches. The hyphae comprising the young colony may be long, as in strain 15 (figure 22) or very short as in strains 9 (figures 10 and 11) and 90 (figures 30 and 31).

Organisms which are morphologically GROUP II form a characteristically branched young colony, and fragmentation is delayed longer than in GROUP I. The species that have been studied in this group present a more homogeneous mycelial and young colony morphology than organisms in GROUP I. It would be very difficult, if not impossible, to characterize many of these species on this basis alone. STRAINS 1, 2, 12, 33, 42, and 74 present a similar colonial morphology. Detection of differences would require studies of individual cells as they develop into young colonies. The similarity of STRAINS 76 (FIGURES 57–60) and 78 (FIGURES 61–64) is striking.

The young colonies of organisms belonging to morphological GROUP III that have been studied present a similar picture, characterized by wide spread and by long branching hyphae which overlap and cross freely, forming a diffuse young colony. Organisms of this group also characteristically form aerial and substrate hyphae. Some cultures present differences, such as the smaller colony produced by STRAIN 52 (FIGURES 85–88). The spirals that were produces by STRAIN 77 (FIGURE 92) were seen in no other culture.

The eight strains of *Nocardia asteroides* were studied to see how much difference there might be in the young colonial morphology of organisms that had been assigned to this species, using current criteria for species determination. STRAINS 84, 101, and 111 showed the greatest differences. STRAINS 92, 93, 94, 95, and 97 were similar morphologically. STRAIN 84 grows in a very characteristic manner in that the hyphae and branches curve suggesting dichotomous branching. The colonies remain small, and there is a limited amount of fragmentation in the center of the colonies. STRAINS 101 and 111 exhibit hyphae which bend and fragment in the center of young colonies, although most strains of *N. asteroides* show no fragmentation up to 90 hours of incubation. These differences in developmental morphology in strains of *N. asteroides* raise fundamental questions as to the importance of this behavior in speciation. Which morphology is "typical" for the species? Are there limits of differences in the developmental morphology of species, or are there species which may show as wide a diversity as is known to exist in the genus? What relationships exist between morphological differences and differences in other behavior? What differences are of most importance in a species concept? In order to answer some of these questions, detailed studies of both morphological and physiological behavior should be made of a large number of isolates of one species.

These studies indicate that the morphology of young developing colonies can be distinctive for individual strains. There are strains in which the morphology of young colonies is not distinctive and could not be distinguished from others on this basis, STRAINS 76 and 78, for example.

Since the fundamental basis of the genus *Nocardia* is a morphological one, a determination of the morphological characteristics of particular species warrants careful study. A determination of the limits of differences of individual cells of a particular isolate as they grow into small colonies should be carefully considered in delimiting species or species groups. Individual isolates can behave distinctively in manner of germination, time of branching, fre-

quency of branching, type of fragmentation, length of branches, and size of colony. Exact studies of these morphological details under constant and reproducible environmental conditions are necessary before use can be made of them in speciation. The reliability of the morphological development of young colonies as criteria for speciation in the genus is therefore yet untested. It will have to await more careful studies of this nature to determine what differences exist between different isolates of the same species, and to studies of correlation between the morphological development of young colonies and other reliable criteria.

At the present time, exact developmental morphological studies seem to offer a rewarding approach to reliable criteria for speciation in the genus *Nocardia*.

Summary

(1) Thirty-seven strains of *Nocardia* species were studied by making observations at 12-hour intervals of cells as they grew to young colonies on agar blocks.

(2) The developmental morphology of some strains are distinctive whereas others are not.

(3) The morphology of young colonies of eight isolates of *Nocardia asteroides* did not all appear alike.

(4) The possibility of using the developmental morphology of young colonies as an aid to speciation is discussed.

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